MOLECULAR CHARACTERIZATION OF THE POST-TRANSLATIONAL REGULATORY MECHANISM OF MOAP-1

BAY WAN PING

NATIONAL UNIVERSITY OF SINGAPORE

2014

MOLECULAR CHARACTERIZATION OF THE POST-TRANSLATIONAL REGULATORY MECHANISM OF MOAP-1

BAY WAN PING

(B. App Sc. (Hons) NUS)

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACY

NATIONAL UNIVERSITY OF SINGAPORE

2014

DECLARATION

I hereby declare that the thesis is my original wok and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

_____

Bay Wan Ping

20th January 2014

ACKNOWLEDGMENTS

First and foremost, I would like to express my gratitude to my supervisor Prof Victor Yu Chun Kong for the opportunity to pursue research in this area. I appreciate his patience, guidance, suggestions and continuous support over the course of this research. I would also like to thank Dr Fu Nai Yang, without his valuable advice, this work would not have been possible.

I would also like to thank our collaborators Prof Wong Hong Rui, for the in-vitro ubiquitination assay work (from Xiamen University, China), Dr Jeffery Wong for the in-vitro kinase assay using [γ-^{32}P] ATP(from IMCB, Biopolis) and Li Rong for the mass spectrometry work (from ETC, Biopolis).

I would like to thank the current and past members of VY lab for their help, support and encouragement during this course of study. Special thanks to Luqi, Chen Wan and Bei Ying who guided and taught me the fundamental techniques that were used throughout my four years of study. I would like to thank Chong Teik for his time and help in making this thesis more readable. I am also grateful to Nala, Qiling and Yu-Chin for always being ready to help and their valuable advice. I would like to express my gratitude to our FYP student, Lynette Lim Shu Qing who assisted me for the kinase inhibitors study. I would also like to thank to Jeremy for his help in processing the purchase orders and to the Department of Pharmacy office staff (Kelly, Timothy and Napsiah) for their assistance on all administrative matters.

Finally, I would like to thank all my family members and my fiancé for loving support during this time of academic challenge.

PUBLICATIONS

1. Arshad, M., Ye, Z. D., Gu, X. F., Wong, C. K., Liu, Y., Li, D., Zhou, L. K., Zhang, Y., Bay, W. P., Yu, V. C., and Li, P. (2013) RNF13, a RING Finger Protein, Mediates Endoplasmic Reticulum Stress-induced Apoptosis through the Inositol-requiring Enzyme (IRE1 alpha)/c-Jun NH2-terminal Kinase Pathway. *Journal of Biological Chemistry* 288, 8726-8736

2. Chen, W., Bay, W. P., Wong, M. W., and Huang, D. J. (2012) Selenium Blue-alpha and -beta: turning on the fluorescence of a pyrenyl fluorophore via oxidative cleavage of the Se-C bond by reactive oxygen species. *Tetrahedron Letters* 53, 3843-3846

TABLE OF CONTENTS

SUMMARY

Bcl-2 protein family regulates the commitment of cells to apoptosis, an ancient cell suicide programme that is vital for development, immunity and tissue homeostasis. While excessive apoptosis augments ischemic conditions and promotes neuro-degeneration, shortage of apoptosis drives cancer and auto-immune diseases. By regulating the activation and oligomerization of Bax and Bak in the outer mitochondria membrane (OMM), the Bcl-2 protein family control the release of apoptogenic factors from mitochondria. MOAP-1, initially named MAP-1 (modulator of apoptosis-1) has previously been cloned as a Bax-associating protein from a yeast-two hybrid screen using Bax as bait. Similar to other Bcl-2 family proteins, MOAP-1 is enriched in the OMM. In healthy cells, MOAP-1 is a short-lived protein ($t^{1/2}$~25mins) which is constitutively degraded by the ubiquitin proteasome system (UPS). Apoptotic stimuli stabilize MOAP-1 and induce its association with Bax in mitochondria. Notably, loss of function analysis suggests MOAP-1 is a critical effector for Bax-mediated apoptosis in mitochondria. Correspondingly, higher levels of MOAP-1 sensitize cancer cells to chemotherapeutic drugs, suggesting mechanisms that are directly involved in regulating its protein stability can potentially be valuable targets for cancer treatment. However, the molecular mechanism of how MOAP-1 sensitizes cancer cells to chemotherapeutic drugs remains unknown.

Post-translational modifications (PTMs) modulate protein function in eukaryotes and have a ubiquitous role in diverse range of cellular functions. An in-depth understanding of the roles of PTMs in modulating protein stability of MOAP-1 is vital not only for gaining perception of a wide array of

potential cellular functions of MOAP-1 but may also be crucial for conceptualizing therapeutic strategies aimed at modulating the protein abundance of MOAP-1. In this study, we examined ubiquitination, phosphorylation and acetylation as prospective PTM regulatory mechanisms of MOAP-1. For the investigation on the ubiquitination of MOAP-1, we show that tripartite motif protein 11 (TRIM11) associates with and degrades MOAP-1 via the UPS-mediated mechanisms. Targeted inactivation of putative TRIM11 E3 activity by alanine substitution on conserved cysteine or histidine residues in the RING domain abolished its ability to poly-ubiquitinate and degrade MOAP-1. Collectively, our data support a role for TRIM11 as a putative E3 ubiquitin ligase of MOAP-1. As for the examination on phosphorylation of MOAP-1, we revealed that protein stability of MOAP-1 is regulated by phosphorylation at serine-27 (Ser-27), Ser-29 and Ser-31 and that over-expression of constitutive active MAPK kinase (MEK1Δ) can up-regulate and stabilize MOAP-1. Constitutive phosphorylation at these three serine residues by aspartate substitution prolongs the half-life of MOAP-1 and is required for MEK1Δ-mediated stabilization of MOAP-1. In agreement with its effect on enhancing MOAP-1 stability, MEK1Δ sensitizes cancer cells to TRAIL-induced Bax-activation and apoptosis. Together, our data support a role for MEK1Δ in modulating the stability of MOAP-1 through phosphorylation at Ser27, Ser-29 and Ser-31. Lastly, we identified SIRT2 and SIRT5, which are members of the sirtuin family of deacetylases, as novel interactors of MOAP-1. Our data support a role for SIRT5 in mediating the regulation of MOAP-1 under DNA-damaging agent, ETOP.

In conclusion, this study explored ubiquitination, phosphorylation and acetylation as prospective PTM regulatory mechanisms for regulating the protein stability of MOAP-1, providing further insight to enhance our understanding of the mechanisms that serve to regulate MOAP-1 stability. Hence, TRIM11, MEK1Δ and SIRT5 may be explored as potential targets to induce up-regulation of MOAP-1 in cancer cells.

ABBREVIATIONS

Acetyl-CoA	Acetyl-coenzyme A
ADP	Adenosine di-phosphate
AIDS	Acquired immuno-deficiency syndrome
AKT	v-Akt murine thymoma viral oncogene
AMP	Adenosine mono-phosphate
AMPK	AMP-activated protein kinase
APAF-1	Apoptotic protease activating factor
APC/C	Anaphase-promoting complex cyclosome
APS	Ammonium persulfate
ATP	Adenosine tri-phosphate
Bad	Bcl-2-antagonist of cell death
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated x protein
Bcl-2	B-cell lymphoma/leukemia-2
Bcl-xL, Bcl-xS transcript	Bcl-2 related protein, L=long transcript, S=short
β-TrCP	Beta transducin-containing protein
BH domain	Bcl-2 homology domain
Bid	BH3-interacting domain death agonist
Bik	Bcl-2-interacting killer
Bim	Bcl-2-like-11
Bmf	Bcl-2 modifying factor
Bok	Bcl-2-related ovarian killer
BSA	Bovine serum albumin
CPS1	Carbamoyl phosphate synthetase 1
Caspase	Cysteinyl aspartate-specific protease
cDNA	Complementary deoxyribonucleic acid

CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
CHX	Cycloheximide
Co-IP	Co-immuno-precipitation
CRL	Cullin-based RING ubiquitin ligase
Cy3	Cyanine 3
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DMSO	Dimethylsulfoxide
dNTP	Deoxynucleotide triphosphate
Dox	Doxycycline
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
DUB	Deubiquitinases
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetate
ER	Endoplasmic reticulum
ERK	Extracellular signals regulated kinase
ETOP	Etoposide
FADD	FAS-associated death domain
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FOXO	Forkhead box O
G418	Geneticin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDH	Glutamate dehydrogenase
GDP	Guanosine di-phosphate

GFP	Green fluorescent protein
GPCRs	G-protein couple receptors
GRB2	Growth-factor-receptor bound protein-2
GSK-3	Glycogen synthase kinase-3
GST	Glutathione-S-transferase
GTP	Guanosine tri-phosphate
HA	Hemagglutinin
HATs	Histone acetyltransferases
HDAC6	Histone deacetylase 6
HDACs	Histone deacetylases
HECT	Homologous with E6-associated protein C-terminus
HEK 293T	Human embryonic kidney cell-line
HeLa	Human cervical adenocarcinoma cell-line
HEPES	N-2-hydroxyethyl peperazine-N'-2-ethanesulfonic acid
HRP	Horseradish peroxidase
HRK	Harakiri (also known as death protein-5)
HSP60	Heat shock protein 60
IAPs	Inhibitors of apoptosis
IMS	Intermembrane space
IP	Immuno-precipitation
IPTG	Isopropyl-β-D-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
Krebs cycle	Tricarboxylic acid cycle (TCA cycle)
LB	Lysogeny broth
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
Mcl-1	Myeloid cell leukemia 1
MEK	MAPK ERK Kinase

MG132	Carbobenzoxyl- leucinyl- leucinyl- leucinal-H
MOAP-1	Modulator of apoptosis
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger RNA
mTOR	Target of rapamycin
NADH	Nicotinamide adenine dinucleotide
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NP-40	Nonidet P-40
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with 0.1% Tween® 20
PCR	Polymerase chain reaction
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator
PDK1	Phosphoinositide-dependent kinase 1
PEPCK1	Phosphoenolpyruvate carboxykinase
PH	Pleckstrin homology
PI3K	Phosphoinositide-3 kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PMSF	Phenylmethylsulfonyl fluoride
PNMA	Paraneoplastic Ma antigens
PP-1	Protein phosphatase 1
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
PUMA	Bcl-2 binding component-3
PVDF	Polyvinylidene difluoride membrane
qRT-PCR	Quantitative real-time polymerase chain reaction
RASSF1A	Ras-associating domain containing protein 1

rDNA	Ribosomal deoxyribonucleic acid
Rheb	Ras homolog enriched in brain
RING	Really interesting new gene
RNA	Ribonucleic acid
RTKs	Receptor tyrosine kinase
Rsk	Ribosomal S6 kinase
siRNA	Small interfering RNA
SDS	Sodium sodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate poly-acrylamide gel electrophoresis
Ser	Serine
SH2	Src homology 2
siRNA	Small interfering ribonucleic acid
SOS	Son-of-sevenless
STS	Staurosporine
TAE	Tris-acetate-EDTA
tBid	Truncated Bid
TEMED	Tetramethylethylenediamine
TIP60	Type 1-interacting protein
TM	Transmembrane domain
TNF	Tumor necrosis factor
THA	Thapsgargin
Thr	Threonine
TRAIL	TNF-related apoptosis-inducing ligand
TRIM	Tripartite motif family
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TSC	Tuberous sclerosis complex
Tyr	Tyrosine
UPS	Ubiquitin-proteasome system
USP9X	Ubiquitin-specific peptidase 9, x-linked

UV	Ultra-violet
WT	Wild type
XIAP	X-linked inhibitor of apoptosis

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

Introduction

1.1 APOPTOSIS

1.1.1 Definition

Apoptosis or programmed cell death, is an essential aspect of life for multi-cellular organisms (1). The term originates from a Greek word "απόπτωσις" which signifies the shedding of leaves from trees in autumn. While the observation was first described by Carl Vogt, a German scientist in 1842, the word "apoptosis" was primarily proposed and coined only in 1972 by three British scientists Kerr J.F., Wyllie A.H. and Currie A.R. to describe a morphologically distinct form of cell death (2). Apoptosis is acknowledged as a critical and distinctive mode of programmed cell death. It plays an essential role in the development of the embryo and the elimination of superfluous, ectopic, damaged or mutated cells in multi-cellular organisms (3). The importance of apoptosis is evidenced with it being a highly conserved process that has evolved across species to maintain cell numbers and cellular positioning within tissues (4-6).

Through the decades, many forms of programmed cell death have been described such as necrosis, necroptosis (programmed necrosis) as well as other forms of programmed cell death yet to be discovered. However, apoptosis is distinctive as it is orderly and does not cause inflammation to neighbouring healthy cells. Typically, cells undergoing apoptosis are characterized by several distinctive morphological and molecular changes (6). Cell shrinkage, membrane surface blebbing, chromatin condensation, DNA fragmentation, externalization of phosphatidylserine and ultimately the breakdown of the cell into membrane-enclosed vesicles known as 'apoptotic bodies' are examples of

characteristic morphological and biochemical features associated with apoptosis (Fig 1.1) (7-11). In addition, a key feature of activated apoptosis is the cleavage of cytoskeletal proteins by cysteine-dependent aspartate-directed proteases (caspases) which thereby results in the collapse of sub-cellular components (12). In most cases, apoptotic bodies are eliminated by neighbouring phagocytic cells. The externalization of phosphatidylserine results in the recruitment of macrophages to engulf these apoptotic bodies. In doing so, inflammatory response and damage to neighbouring healthy cells are prevented or at least reduced (13).

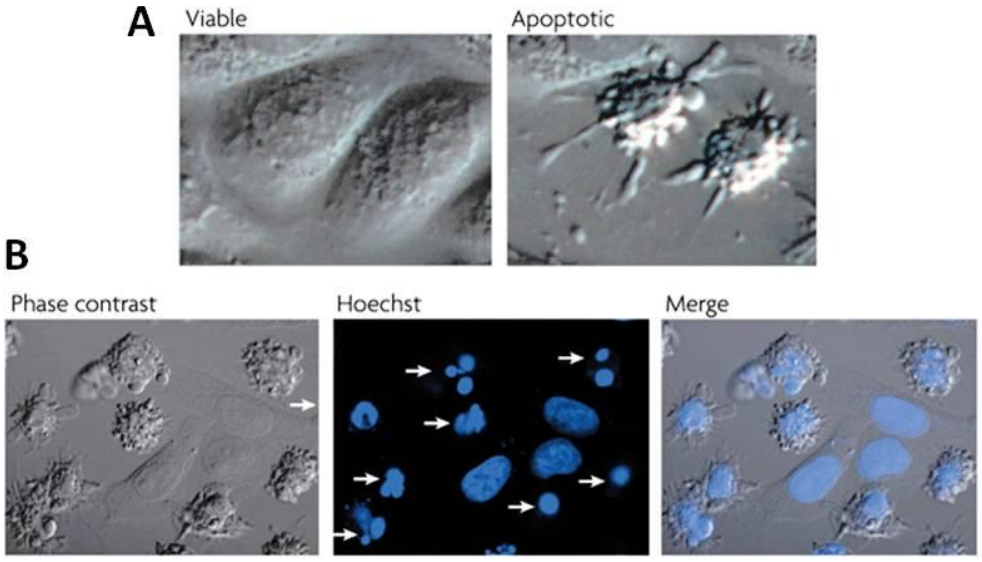

Figure 1.1 Morphology of apoptotic cells. A. The left panel shows healthy HeLa cells while the right panel shows cells displaying characteristic apoptotic morphology including cell shrinkage and plasma membrane blebbbing. **B.** Features of apoptotic nuclear condensation and fragmentation. HeLa cells were induced to undergo apoptosis followed by staining of the nuclei (blue) with Hoechst dye. For comparison, a mixture of healthy and apoptotic cells is shown. Apoptotic cells (indicated with white arrows) exhibit extensive plasma membrane blebbing and contain nuclei that are condensed and/or fragmented into several pieces. Figure is reproduced and adapted from (14).

Greater understanding of apoptosis was achieved from the analysis of programmed cell death during the development of the nematode *Caenorhabditis elegans (15)*. In this organism, 1090 somatic cells are created in the development of the adult worm, of which 131 of these cells will undergo apoptosis. The precise death of these 131 cells demonstrates the extraordinary accuracy and precise control in this process. It is not surprising then that improper functioning of apoptosis signaling has been linked to a plethora of human ailments, ranging from neuro-degenerative disease to cancer (16).

1.1.2 Apoptotic cell death and diseases

Increased apoptosis has been associated with and contributed to acute ischemic diseases associated with reperfusion injury, such as myocardial infarction, stroke and renal hypoxia (17,18). In neurological disorders including, but not limited to, Alzheimer's, Parkinson's and Huntington's diseases, specific neurons prematurely commit suicide which can result in irreversible memory loss, uncontrolled muscular movements and depression (19,20). Involvement of increased apoptosis in arteriosclerosis, infertility, AIDS, diabetes and hepatitis have also been reported (18,21). Decreased apoptosis is known to be involved in cancer and auto-immune disorders. In many forms of cancers, key pro-apoptotic proteins are mutated and/or anti-apoptotic proteins are frequently up-regulated leading to the accumulation of cells and the inability of these cancer cells to response well to chemo-therapeutic agents (22). Since effective chemotherapy depends on the proper induction of apoptosis, cancers with defects in the apoptosis signaling pathways are particularly difficult to treat. Over the years, research into

apoptosis has generated much enthusiasm that key proteins in the apoptosis signaling pathway that are targetable by small molecular weight compounds could be promising drug targets to selectively induce apoptosis in cancer cells (12).

1.1.3 Extrinsic and intrinsic pathways of apoptosis

Higher eukaryotes possess two major pathways for initiating apoptosis: the cell-extrinsic death pathway involving death receptors and the cell-intrinsic pathway that is regulated by Bcl-2 family of proteins (Fig 1.2). The former is activated by death-inducing ligands binding to cell surface receptor while the latter is activated by diverse intrinsic signals converging at the mitochondria. Both pathways involve the initiation and amplification of caspase cascades (23). Caspases are the key effectors of apoptosis. They are normally present in healthy cells as zymogens or pro-enzymes with low or no enzymatic activity and require proteolytic activation at aspartate sites to produce the mature, catalytically active forms (24). As illustrated in Fig 1.2, the initiation of early caspase activation is different between the extrinsic death-receptor-mediated and intrinsic mitochondrial-mediated pathways. Although in certain cell types, the extrinsic cell death-receptor-mediated pathway does not require mitochondria involvement to activate caspases, the extrinsic and intrinsic pathways do converge at the mitochondria in most cell types, underscoring the broad and central role serves by the mitochondria in apoptosis execution in mammals (25). For that reason, the mitochondria play a central role in controlling apoptosis events by integrating up-stream apoptosis-inducing signals and regulating the release of apoptogenic factors such as cytochrome c and diablo homologue (SMAC/DIABLO).

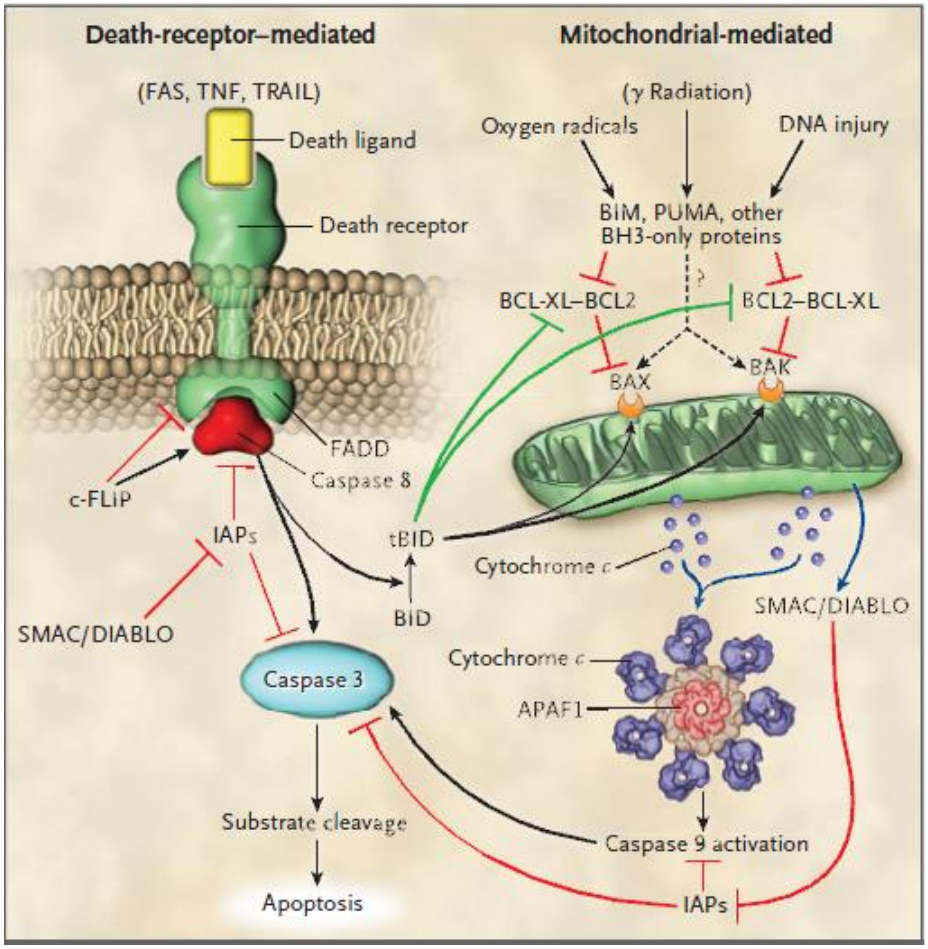

Figure 1.2 Extrinsic and intrinsic pathways of cellular apoptosis. There are two major pathways of apoptosis: the death-receptor pathway, which is mediated by the activation of death receptors, and the Bcl2-regulated mitochondrial pathway, which is mediated by noxious stimuli that ultimately lead to mitochondrial injury. Ligation of death receptors recruits the adaptor protein FAS-associated death domain (FADD). FADD in turn recruits caspase 8, which ultimately activates caspase 3, the key effector caspase. In contrast to the extrinsic death-receptor-mediated pathway utilized by a limited set of death stimuli, the intrinsic mitochondrial-mediated pathway integrates a broad range of extra-cellular and intra-cellular stresses such as DNA damage, unbalanced proliferative stimuli and nutrient or energy depletion (26). In the intrinsic mitochondrial-mediated pathway, pro-apoptotic BH3-only proteins are activated by noxious stimuli which interact with and inhibit anti-apoptotic Bcl-2 or Bcl-xL. Thus, Bax and Bak are free to induce mitochondrial permeabilization with the release of cytochrome c. Cytochrome c released from the mitochondria binds to the adaptor molecule apoptotic protease activating factor (APAF-1) in the presence of ATP to form a large complex term apoptosome which results in the activation of caspase 9. Caspase 9 then activates caspase 3. SMAC/DIABLO is also released after mitochondrial permeabilization and acts to block the action of inhibitors of apoptosis protein (IAPs such as XIAP) which inhibit caspase activation. In addition, the truncated form of Bid (tBid) which is generated by caspase 8-mediated proteolysis of BID in the extrinsic death receptor-mediated pathway, can engage the mitochondria pathway to amplify the apoptotic response. This amplification mechanism is required for effective apoptosis in certain cells (denoted ‘type 2’ cell) such as hepatocytes but not in ‘type 1’ cells such as thymocytes (27,28). The level of XIAP distinguishes the two cell types as it is typically higher in type 2 cells. The figure is reproduced and adapted from (12).

1.2 BCL-2 FAMILY PROTEINS: LIFE/DEATH SWITCH IN MITOCHONDRIA

1.2.1 Mitochondria as the central organelle for apoptosis regulation

Mitochondria are ubiquitous membrane-enclosed organelles that undertake multiple critical functions in a cell. They exert both vital and lethal functions in physiological and pathological scenarios (29). For instance, while mitochondria are indispensible for energy production and hence for the survival of eukaryotic cells; they are also the key regulators of the intrinsic pathway of apoptosis (30). Mitochondrial control the activation of apoptotic effect mechanisms by regulating the translocation of apoptogenic factors from the mitochondrial intermembrane space to the cytosol (Fig 1.3) (29).

Besides their role in apoptosis and energy production, the mitochondria also regulate calcium homeostasis and host several other metabolic circuitries including, but not limited to, the Krebs cycle, the urea cycle, gluconeogenesis, ketogenesis, heme biosynthesis, fatty acid β-oxidation, steroidogenesis and amino acids metabolism. As the mitochondria play critical roles in numerous bio-energetic, anabolic and cell-death-inducing biochemical pathways, it is not surprising that the mitochondrial dysfunction have been contributed to the development of a plethora of human diseases which range from highly tissue-specific conditions to generalized whole-body disorders including cancer (31,32). In the same context, multiple hallmarks of cancer cells including, but not limited to, proliferative potential, resistance to anti-growth signals, impaired apoptosis and decreased autophagy have been correlated with mitochondrial dysfunction (33).

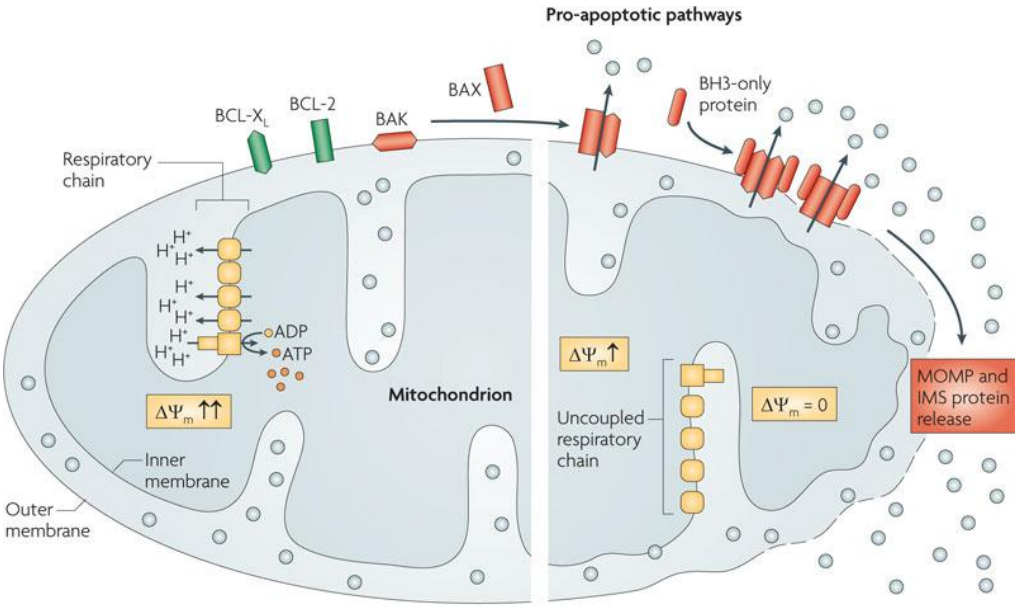

Figure 1.3 Schematic illustration of the mitochondrion and its role in regulating apoptosis. Mitochondria contain inner and outer membranes composed of phospholipid bilayers and proteins. The outer mitochondrial membrane encloses the entire organelle and contains porins that are involved in the transport of molecules ≤ 5kDa. Larger molecules require active transport by mitochondrial membrane transport proteins. The inner mitochondrial membrane is compartmentalized into numerous cristae which increase the surface area of the inner mitochondrial membrane and hence maximize its ATP generation capacity (34). Under physiological conditions, the mitochondria exhibit a high mitochondrial transmembrane potential ($\Delta_{\psi m}$) which is generated by the respiratory chain and exploited for ATP production via oxidative phosphorylation. However, in the presence of apoptotic signals, pro-apoptotic BAX and BAK oligomerization results in the dissipation of the $\Delta_{\psi m}$ and in the osmotic swelling of the mitochondrial matrix which eventually leads to mitochondrial outer membrane permeabilization (MOMP) and the release of apoptogenic factors normally confined within the mitochondrial intermembrane space (IMS) into the cytosol. Once in the cytosol, these factors activate caspase-dependent and –independent mechanisms that altogether mediate the execution of cell death. The figure is reproduced and adapted from (29).

In 1972, Kerr *et al.* proposed in their seminal paper that the mitochondria appeared normal during apoptosis and hence it was initially believed that apoptosis was controlled at the nuclear level (35). This belief was further supported by evidence showing that cells lacking mitochondrial DNA are still able to under apoptosis (36). However, indirect evidence seems to suggest a possible role of mitochondria in apoptosis. Early investigation on

the Bcl-2 protein demonstrated that Bcl-2 localized in the mitochondria and the lack of the C-terminal transmembrane domain was found to diminish its ability to inhibit apoptosis (37,38). However, the earliest direct evidence demonstrating the involvement of mitochondria in apoptosis was conducted in a cell-free model system by Reed and colleagues in 1994 (39). The authors discovered that a dense organelle fraction enriched in mitochondria from Xenopus egg extracts was able to induce chromatic condensation, shrinkage and fragmentation of the nuclei, all of which were characteristic hallmarks of apoptosis. Shortly, mitochondrial permeability transition was published as the point-of-no-return, a critical event leading to cell death via intrinsic apoptosis and subsequently, the involvement of mitochondria in apoptosis was proposed (40,41). A breakthrough in the understanding of the involvement of mitochondria in apoptosis was the discovery of the release of cytochrome c from the mitochondria resulting in the initiation of the caspase cascades (42,43). This finding strongly established an active role of mitochondria in the regulation of apoptosis signaling pathway and the molecular mechanism of apoptotic cell death.

1.2.2 Regulation of mitochondrial outer membrane permeabilization by Bcl-2 family of proteins

While the molecular networks and mechanisms regulating apoptosis signaling in mitochondria are still being actively investigated, the B-cell lymphoma (Bcl-2) family of proteins is widely recognized as the key regulators in the intrinsic mitochondrial-mediated apoptosis pathway. The Bcl-2 family which includes 17 or more members in mammalian cells (16) functions as ‘life/death switch’ that integrates diverse inter- and intra-cellular

signals to determine whether or not the stress apoptosis pathway should be activated (44). They are characterized by the presence of several conserved motifs known as the Bcl-2 homology (BH) domains (45). The Bcl-2 family comprises of three major subfamilies; the multi-domain anti-apoptotic, pro-apoptotic and the BH3-only pro-apoptotic proteins (Fig 1.4) in which interactions between proteins within the three subfamilies will regulate the 'life/death switch'.

In a healthy cell, the pro-apoptotic proteins such as Bax and Bak are kept in check by the pro-survival Bcl-2 proteins. Bcl-2, Bcl-xL and Mcl-1 are the major members of the anti-apoptotic Bcl-2 repertoire and they preserve the outer mitochondrial membrane integrity by direct sequestration to neutralize the pro-apoptotic Bcl-2 proteins (46,47). For instance, the BH1 to BH3 domains of Bcl-xL are in close proximity and create a hydrophobic pocket that can accommodate a BH3 domain of the pro-apoptotic proteins, thereby preventing them from perturbing the integrity of the outer mitochondrial membrane.

However, when the cell is subjected to death-inducing stimuli such as DNA damage, growth factor deprivation or endoplasmic reticulum (ER) stress, the pro-apoptotic BH3-only proteins are activated. The BH3-only proteins function as damage sensors by acting as antagonists of Bcl-2 and other anti-apoptotic proteins. Depending on the individual BH3-only protein, some such as Bad and Noxa bind to the anti-apoptotic Bcl-2 repertoire and thus inhibiting the anti-apoptotic function of these proteins (48-50) while others such as tBid, Puma and Bim are capable of directly inducing Bax and Bak activation (48-54). Undeniably, triple genetic deletion of *Bid*, *Bim* and *Puma* abolished the

homo-oligomerization of Bax and Bak and thereby the cytochrome c-mediated activation of caspases in response to diverse death signals suggesting that the activation of Bax and Bak is fully dependent on Bid, Bim and Puma (54). An important biochemical difference between Bax and Bak is that in healthy cells, Bax is largely cytosolic or loosely associated with mitochondria while Bak is an integral membrane protein on the cytosolic face of the mitochondrion and ER. Hence, in response to cytotoxic signals, only Bax will translocate to the mitochondria whereby both Bax and Bak will undergo conformational changes to form membrane-associate homo-oligomers appearing as foci on the mitochondria (55,56). Remarkably, either action by the BH3-only proteins will facilitate homo-oligomerization of pro-apoptotic multi-domains Bax and Bak into the pore within the outer mitochondrial membrane, thereby promoting mitochondrial outer membrane permeabilization (MOMP) and ultimately apoptotic cell death (57,58). Thus the interplay among the Bcl-2 family proteins is crucial for Bax/Bak activation and the subsequent commitment of intrinsic mitochondria-mediated apoptosis (59-61).

Murine embryonic fibroblasts (MEFs) with Bax or Bak deletion displayed no defect in apoptosis but MEFs with Bax and Bak double knockouts, surprisingly showed dramatic resistance to diverse apoptotic stimuli, suggesting Bax and Bak are central, but redundant effectors of mitochondrial-mediated apoptotic signaling (62). Analysis on apoptosis signaling events in human cell lines and tumor tissues, however, suggests that Bax may exert a dominant function over Bak in human context (63,64).

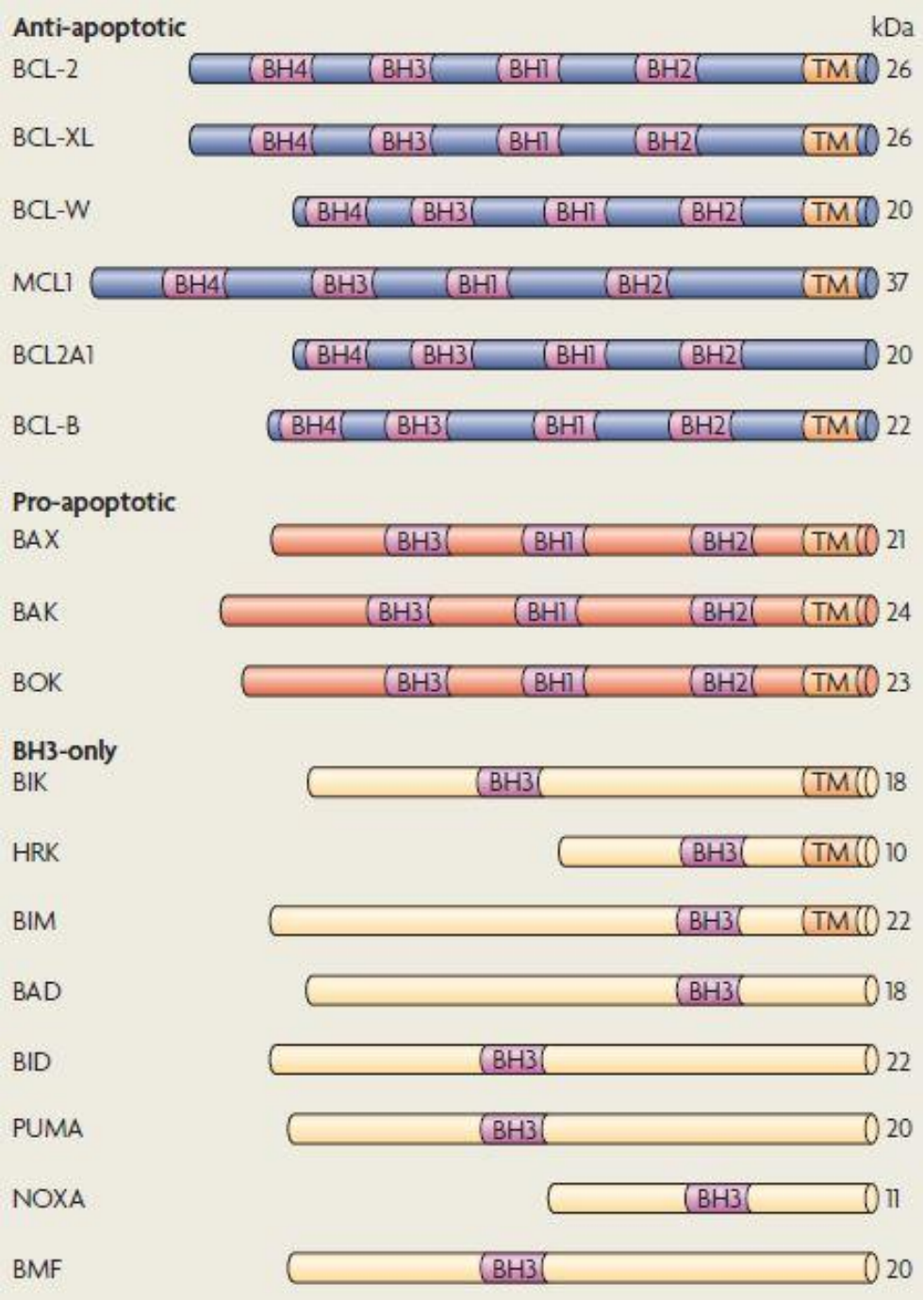

Figure 1.4 Classification of the Bcl-2 family of proteins based on conservation of BH domains. The three main subfamilies are indicated as anti-apoptotic, pro-apoptotic and BH3-only. BH1 to BH4 are the four conserved sequence motifs. Multi-domain pro-apoptotic proteins and anti-apoptotic members share BH 1-3 domains while the BH3-only proteins contain only a single domain of homology. The figure is reproduced and adapted from (14).

1.2.3 Identification of MOAP-1 as a critical effector of Bax-mediated apoptosis

MOAP-1, initially named as MAP-1 (modulator of apoptosis 1) was identified as a binding partner of Bax in a yeast two hybrid screen by our laboratory more than a decade ago (65). Notably, MOAP-1 is also found to belong to the Paraneoplastic Ma antigens (PNMA) family which comprises of six members, PNMA1-6 in human and is designated as PNMA4. MOAP-1/PNMA4 was first studied independently from the PNMA family and it is the best characterized member up to date. Remarkably, similar to other members of the Bcl-2 family of proteins, endogenous MOAP-1 is found to be enriched at the outer membrane of mitochondria (66). While northern blot analysis showed that mRNA of *MOAP-1* is ubiquitously expressed in most organs, the protein level of MOAP-1 is only readily detected in the mouse tissues of brain and testis. (65). Subsequent works revealed that MOAP-1 is a short-lived protein with a half-life of approximately 25 mins (Fig. 1.5A) and is constitutively degraded by the ubiquitin proteasome system (UPS) under non-apoptotic condition (Fig. 1.5B) (67). On contrary, apoptotic stimuli induce the stabilization of MOAP-1 (Fig. 1.5C) and its integration and association with Bax in mitochondria (Fig. 1.5D) (66). Functional analysis showed that knock-down of MOAP-1 by siRNA in tumour cell-lines conferred inhibition of apoptotic signaling triggered by multiple apoptotic stimuli and promoted their growth in anchorage-independent fashion (Fig. 1.5E) (66). Similarly, isolated mitochondria from the MOAP-1 knockdown cells were markedly resistant to Bax-induced cytochrome c release (Fig. 1.5F) (65). Taken together, these data support the notion that MOAP-1 is a critical facilitator of Bax-mediated

apoptosis signaling in mitochondria (66). Interestingly, MOAP-1 has also been shown to be a binding partner of tumor suppressor protein, Ras associating domain-containing protein 1 (RASSF1A) (68). RASSF1A specifically links cell death receptor and activated Ras-mediated signaling to Bax activation through MOAP-1. However the precise mechanism of how RASSF1A facilitate the interaction between MOAP-1 and Bax remains to be further investigated. Although MOAP-1 has been demonstrated to play a significant role in modulating Bax-mediated apoptosis, further exploratory investigation will be required to evaluate its role in other cellular aspects such as cell cycle, autophagy or inflammation. Likewise, cellular factors that regulate the protein stability of MOAP-1 are not yet fully elucidated.

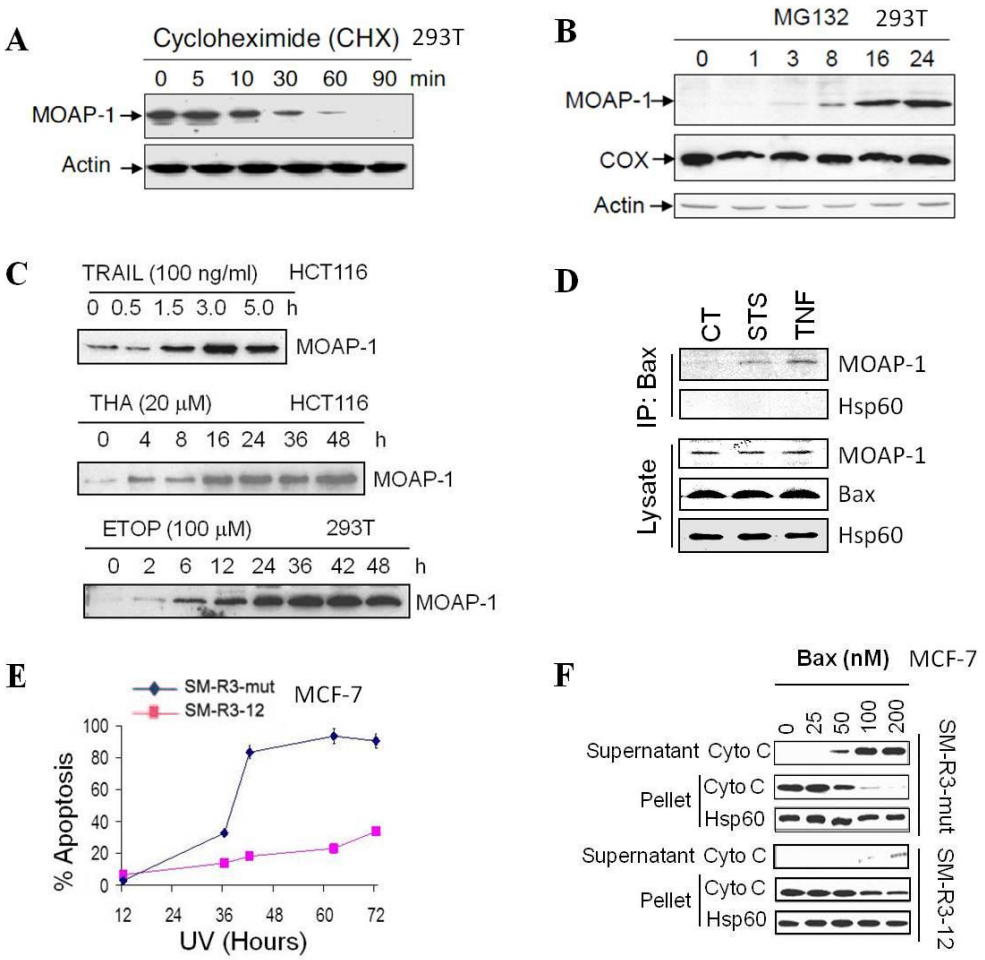

Figure 1.5 Known regulation and function of MOAP-1. A. Inhibition of *de novo* protein synthesis caused rapid elimination of endogenous MOAP-1. **B.** Proteasomal inhibition by MG132 resulted in the up-regulation of endogenous MOAP-1. **C.** Levels of endogenous MOAP-1 protein were rapid elevated by various apoptotic stimuli. **D.** Apoptotic stimuli promote endogenous MOAP-1-Bax association. **E.** MOAP-1 knockdown MCF-7 cells are resistance to apoptotic death triggered by UV. **F.** MOAP-1 is required to facilitate Bax-mediated release of cytochrome c from isolated mitochondria. The data were reproduced and adapted from (66,67).

1.3 POST-TRANSLATIONAL MODIFICATIONS (PTMs) REGULATE PROTEIN ABUNDANCE AND FUNCTION

Signaling pathways that regulate cell survival and cell death are complex. The cellular survival or apoptotic response is often determined by the expression levels, post-translational modifications (PTMs) and protein-protein interaction profiles influencing the function of Bcl-2 protein family members (69). PTMs including, but not limited to, ubiquitination, phosphorylation and acetylation represent highly effective ways for reversible and rapid alteration of the cellular function of a protein (69,70). Multi-site PTMs of proteins modulate protein intrinsic functional activity, folding and conformation, oligomerization state, stability and half-life, turnover rate, sub-cellular localization and protein-protein interactions (69,71). For instance, poly-ubiquitination may mark and target proteins for degradation through the ubiquitin proteasome system (UPS) while phosphorylation may determine whether a protein is recognized and targeted by its E3 ubiquitin ligase for degradation via the UPS. In general, majority of the PTMs target specific amino acids at specific recognition motifs and will often involve the covalent attachment of a specific chemical moiety to the respective amino acid residues on proteins. For instance, ubiquitination and acetylation target the lysine residues while phosphorylation results in the covalent attachment of a

phosphate group to the serine, threonine and/or tyrosine residues of a protein. Interestingly, cells of multi-cellular organisms have extensive networks of PTMs, whereby different modification pathways converge in signal integration. Moreover, the functional and inter-connected network of multiple PTMs between distinctive proteins orchestrates the overall cellular response by fine-tuning the functional activity of proteins (69). Depending on the proteins involved and the cellular context, PTMs can result in either the promotion or inhibition of apoptosis (69). The dynamic interplay between the pro-apoptotic and anti-apoptotic family members is tightly controlled so as to ensure the survival or death of a cell within the appropriate cellular context.

Like many proteins, the abundance and function of Bcl-2 family proteins are regulated by PTMs. It is generally accepted that the molecular regulation of a protein is linked to a highly orchestrated cascade of phosphorylation/dephosphorylation events that are intermingled with ubiquitination/de-ubiquitination, acetylation/deacetylation and other PTMs. One example is that of the molecular regulation of Mcl-1 (myeloid cell leukemia 1), an anti-apoptotic Bcl-2 family protein. Being anti-apoptotic, Mcl-1 suppresses the progression of apoptosis by directly associating with and sequestering pro-apoptotic multi-domain proteins Bak and Bax, deterring the homo-oligomerization of Bax/Bak into the outer mitochondrial membrane thereby preventing MOMP and the release of apoptogenic factors such as cytochrome c into the cytosol. Mcl-1 also binds to and sequesters pro-apoptotic BH3-only Bim which acts as an activator for Bax and Bak. Notably, Mcl-1 was reported to be rapidly regulated and degraded by the poly-ubiquitination-mediated degradation pathway [also known as the ubiquitin

proteasome system, (UPS)] (72). Interestingly, phosphorylation of Mcl-1 by GSK-3 promotes its targeting and poly-ubiquitination by its E3 ubiquitin ligase, β-TrCP (73). Conversely, Mcl-1 has also been shown to be the target of a deubiquitinases, named USP9X, which results in the stabilization of Mcl-1 by reversing its poly-ubiquitination thereby preventing its degradation through the UPS (74). These findings support the notion that PTMs work cooperatively on Bcl-2 family proteins such as Mcl-1 to regulate their function thereby promoting cell survival or apoptosis depending on the cellular context and the nature of the cell survival or cell death stimuli.

1.3.1 Ubiquitination

1.3.1.1 Poly-ubiquitination-mediated protein degradation

Ubiquitin is a highly evolutionarily conserved 76 amino acid residue that is ubiquitously expressed in all eukaryotic tissues. Ubiquitination or the conjugation of ubiquitin to the protein substrate proceeds via a three-step cascade enzymatic mechanism involving E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligases (Fig 1.6) (75). In a ubiquitin, there are seven lysine residues to which free ubiquitin can be conjugated, thus giving rise to seven different linkages (76). In addition, ubiquitin molecules may form a linear chain through their N-terminal methionine residue (Fig 1.6a). Mono-ubiquitination and multiple mono-ubiquitination normally mediate membrane protein endocytosis and endosomal sorting (77,78). For poly-ubiquitination, poly-ubiquitination chains formed via the lysine 48 of the ubiquitin (K48-linked poly-ubiquitination, Fig 1.6b) is the most commonly observed linkage which results in the degradation

of proteins via the ubiquitin-proteasome system (UPS) (Fig 1.7) (79). Remarkably, it was estimated that about 80-90% of intracellular proteins are degraded by the UPS with the remaining 10-20% degraded by the lysosome (80,81).

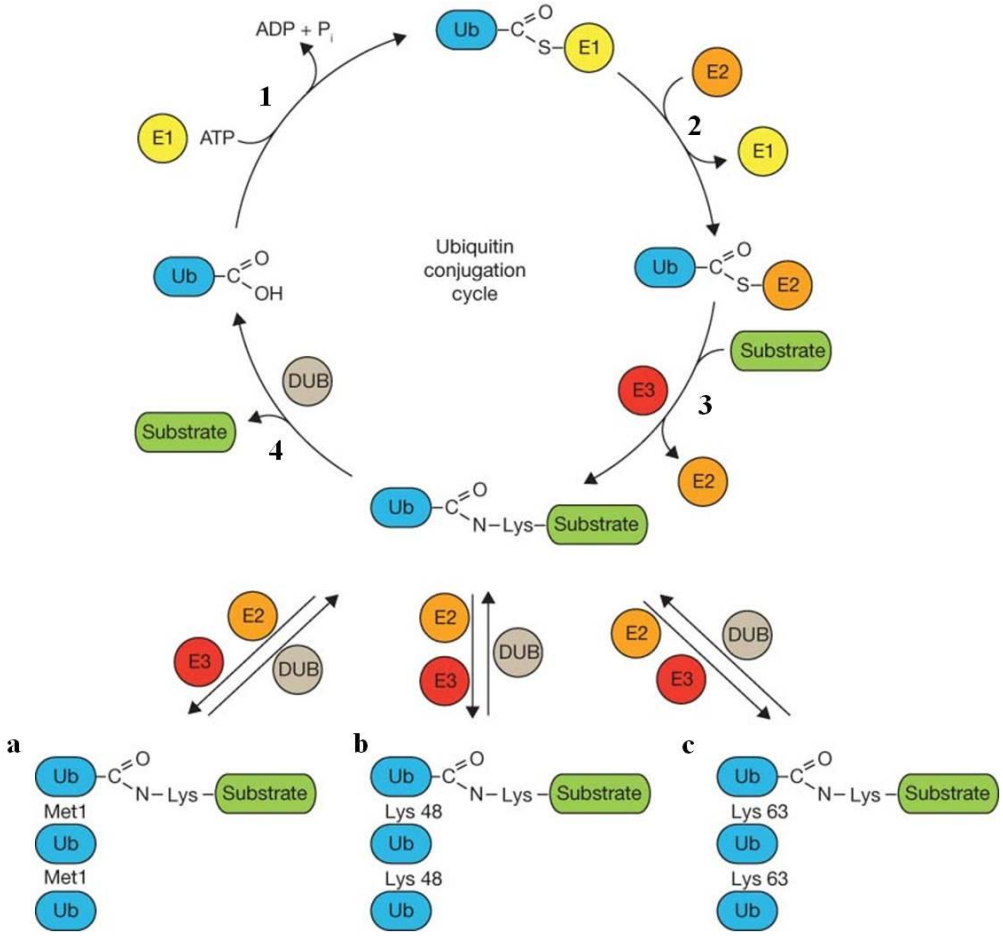

Figure 1.6 An overview of the multi-step enzymatic reaction of ubiquitination. In step (1), an ubiquitin molecule (Ub) is activated by an E1 ubiquitin-activating enzyme, resulting in the conjugation of the ubiquitin to a cysteine residue via a thioester bond in the active site of the E1 enzyme. This step requires the hydrolysis of an adenosine tri-phosphate (ATP), which is converted into an adenosine di-phosphate (ADP) and phosphate group, releasing energy to drive this E1-ubiquitin conjugation. In step (2), the E1 transfers the ubiquitin to a cysteine residue in the active site of the E2 enzyme. In step (3), the E2 ubiquitin conjugate then binds to an E3 ubiquitin ligase which recognizes its specific substrate protein and serves as a scaffold to allow the transfer of ubiquitin from the E2 to (usually) a lysine residue in the protein substrate (for RING-type E3s) or catalyzes the transfer via its ligase domain (for HECT-type E3s). The first three steps can be repeated to form a poly-ubiquitin chain on the substrate. The generation of poly-ubiquitin chains can occur at any one of the seven ubiquitin lysine residues and E2 conjugating enzymes may help to specific the linkage type in poly-ubiquitin chains. **(a)** Linear ubiquitination: Ubiquitin molecules may form a linear chain through their N-terminal methionine (Met1) (82). **(b)** K48-linked poly-

ubiquitination: Poly-ubiquitination chains formed via the lysine (Lys) 48 of the ubiquitin usually target proteins for degradation via the ubiquitin proteasome system (83-85). **(c)** K63-linked poly-ubiquitination: Poly-ubiquitination chains formed via the lysine 63 of the ubiquitin target proteins for lysosomal degradation and other non-proteolytic roles such as membrane protein endocytosis, DNA repair and activation of protein kinases (77,86-88). This type of modification does not appear to involve proteolysis of the target substrates but plays a role in the activation/inactivation of the target protein. Lastly in step (4), deubiquitinases (DUB) may disassemble the ubiquitin chains, removing the ubiquitin molecules from the protein substrates (75). Figure is modified and adapted from (89).

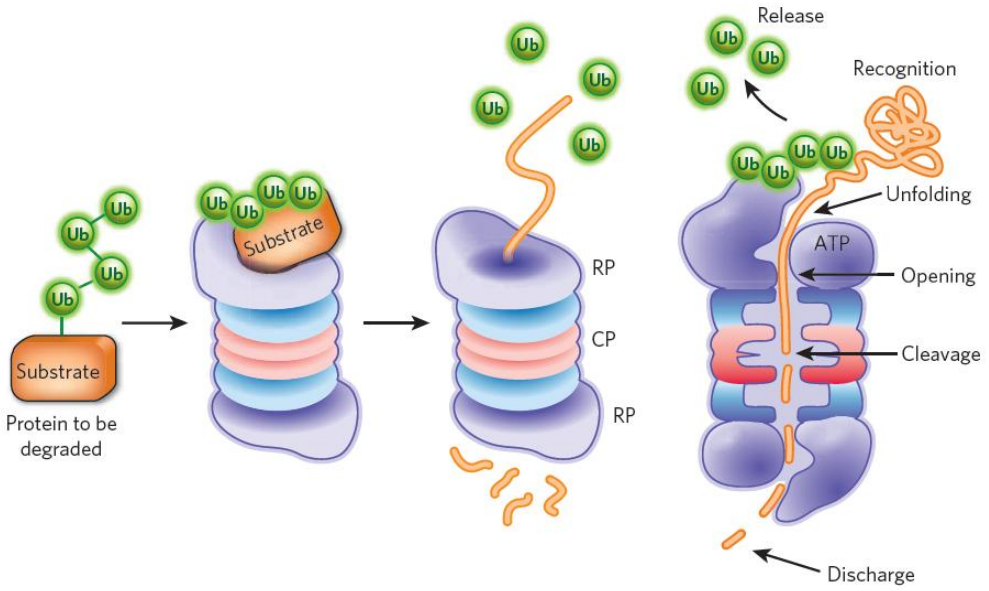

Figure 1.7 Schematic representation of the ubiquitin-proteasome system (UPS). Degradation of poly-ubiquitinated substrates is carried out by a large multi-protein complex referred to as the 26S proteasome (80). It is composed of a 20S catalytic core (CP) formed by four rings containing two types of catalytic subunits (α and β) and two 19S regulatory subunits (RP) (80,90). The regulatory subunit consists of multiple ubiquitin binding proteins (also known as ubiquitin receptors), ATPases, deubiquitinases and other adaptor proteins. Upon recognition by the ubiquitin receptors, ubiquitinated substrates are unfolded by ATPases and deubiquitinases reverse the covalent conjugation, resulting in the release of free ubiquitin (Ub) for recycling. The unfolded substrates are subsequently translocated into the 20S catalytic core of the proteasome through the opening of a narrow gated channel. The catalytic core contains a high number of proteolytic active sites that cleave the protein substrate into small peptides. The small peptides are then discharged from the base of the proteasome. Figure is reproduced and adapted from (91).

1.3.1.2 E3 ubiquitin ligases

E3 ubiquitin ligases are key regulatory elements of the UPS as they confer substrate specificity and catalyze the last step in the conjugation of the ubiquitin to the substrate. Typically, the human ubiquitin system is believed to encode two E1s, approximately 50 E2s and over 500 E3s, forming an E1-E2-E3 hierarchical pyramid (80,89). Based on the sequence homology of their E2-binding domains, E3 ubiquitin ligases are mainly categorized into two main groups: HECT (homologous with E6-associated protein C-terminus)-type E3 ubiquitin ligases and RING (Really Interesting New Gene)-type E3 ubiquitin ligases (Fig 1.8). The RING-type E3 ubiquitin ligases are the most abundant E3 ligases identified in eukaryotes to date (92,93) and they are known as RING-type E3 ubiquitin ligases due to the possession of a catalytic RING finger domain (94). Similarly, the HECT-type E3 ubiquitin ligases are characterized on the basis of their large C-terminal HECT domain which is a protein module of approximately 350 amino acids that was first identified in the E6-associated protein (E6-AP) (95). Possessing the HECT domain as the catalytic domain, HECT-type E3 ubiquitin ligases have intrinsic catalytic activity and can interact with E2 ubiquitin-conjugating enzymes and protein substrates directly without the need for adaptor or auxiliary proteins (95). The major difference between RING-type and HECT-type E3 ubiquitin ligases is that RING-type E3 ligases mediate the direct transfer of the ubiquitin straight from the E2 ubiquitin-conjugating enzymes to the protein substrates whereas the HECT-type E3 ubiquitin ligases first transfer the ubiquitin to themselves before conjugating it to the substrates (95,96).

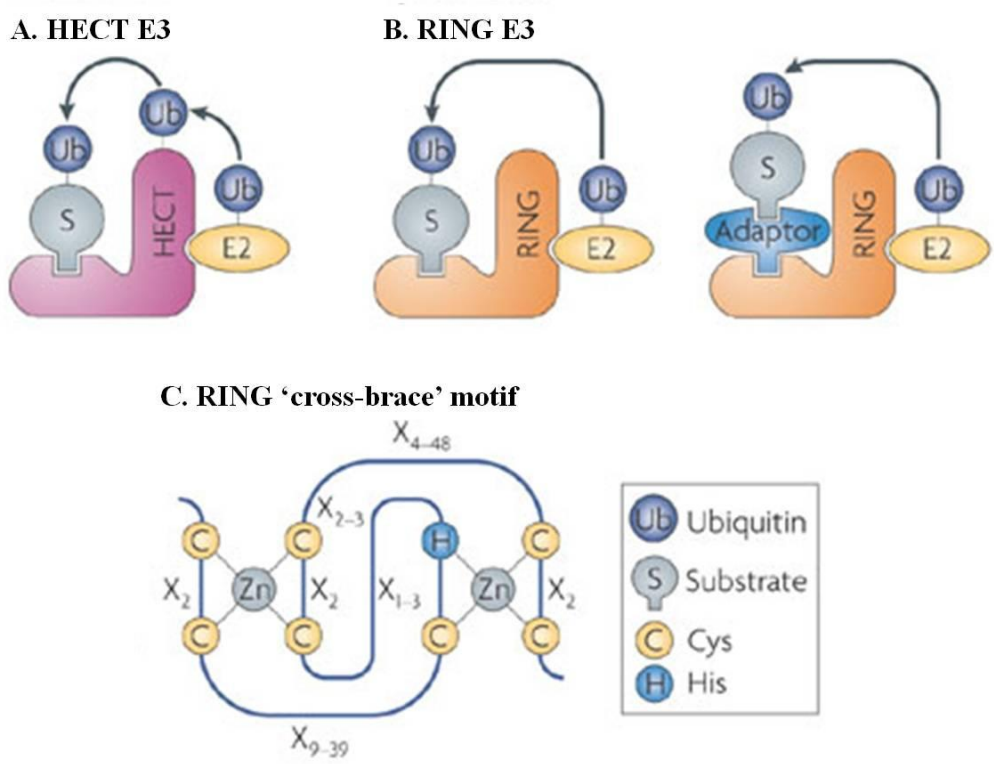

Figure 1.8 Two main types of E3 ubiquitin ligases. (A) HECT-type E3 ubiquitin ligase transfer ubiquitin from E2 ubiquitin-conjugate enzymes to a cysteine residue in their active sties before conjugating the ubiquitin onto the protein substrate. HECT-type E3 ligases normally function on their own and they contain domains that allow interaction with E2 enzymes and substrate recognition domains that allow binding to their specific targeted protein substrates. **(B)** RING-type E3 ubiquitin ligases typically serve as a scaffold, bringing the substrate proteins in close proximity to the E2 ubiquitin-conjugating enzymes, thereby mediating the direct transfer of ubiquitin from the active sites of E2 enzymes to the protein substrates. **(C)** RING-type E3 ubiquitin ligases are characterized by a RING domain which produces a 'cross-brace' motif that is stabilized by the biding of two zinc atoms through cysteine and histidine residues. Figure is modified and adapted from (97).

1.3.1.3 TRIM family of proteins as E3 ubiquitin ligases

Notably, the RING finger motif is defined as a unique linear series of conserved cysteine and histidine residues. As illustrated in Fig 1.8c, the RING finger motif is composed of a unique "cross-brace" arrangement with two zinc ions and it folds into a compact domain comprising of a small central β sheet and an α helix (98,99). Frequently, the RING domain is associated with

cysteine-rich B-box domains followed by a predicted coiled-coil domain. This ensemble of a RING domain, one or two B-box domains and a coiled-coil domain are called RBCC or TRIM (100). Indeed, studies have reported RBCC/TRIM family of proteins functioning as E3 ubiquitin ligases with specific substrates identified. For instance, TRIM2 was identified as the E3 ubiquitin ligase that ubiquitinates cell death-promoting protein Bcl-2-interacting mediator of cell death (Bim) (101). It was previously reported that phosphorylation of the serine 65 residue by ERK1/2 is a critical event in the degradation of Bim (102). Notably, the ubiquitination and degradation of Bim by TRIM2 are blocked by inhibition of the MAPK cascade with MEK inhibitor, U0126 (refer to Section 1.3 for discussion on the MEK-ERK MAPK cascade). This example demonstrates the interplay between two PTMs in which phosphorylation of a protein permits the subsequent poly-ubiquitination, which is required for recognition and degradation by the UPS. In addition, TRIM proteins have also been reported to regulate the stability of other E3 ubiquitin ligases, thereby modulating the protein abundance of substrates of the targeted E3s. For example, TRIM39 was previously demonstrated by our laboratory to be a binding partner of MOAP-1 that stabilizes MOAP-1 through inhibition of its poly-ubiquitination process (103). It was later revealed by another group that the influence of TRIM39 on MOAP-1 stems from the ability of TRIM39 to directly inhibit APC/C^{Cdh1}-mediated MOAP-1 ubiquitination (104).

1.3.2 Phosphorylation

1.3.2.1 Phosphorylation, a major protein regulatory mechanism

Phosphorylation, a widely exploited PTM used in signal transduction to control cellular homeostasis (105), is thus far the most commonly reported protein modification that occurs in mammalian cells (106). Functioning as a form of spatial and temporal regulation on proteins, phosphorylation shares similarities with ubiquitin conjugation in regards to its reversibility. Remarkably, nearly 30% of all eukaryotic cellular proteins are phosphorylated and chromosomal mapping indicates that 244 of 518 kinase genes are disease- or cancer- related, demonstrating its ubiquity and importance in signal transduction (70,107). Phosphorylation involves the transfer of the terminal phosphate of adenosine tri-phosphate (ATP) to a hydroxyl group of an amino acid principally on serine (Ser), threonine (Thr) or tyrosine (Tyr) residues of most proteins (Fig 1.9). Because each phosphate group carries two negative charges, the addition of a phosphate group may change the protein conformation and consequently the functional activity of the protein. In this context, the ability of phosphorylation to alter the conformation of a protein allows for regulated control on the activity of the protein.

Enzymes that phosphorylate proteins (protein kinases) and those that remove phosphates (protein phosphatases) usually activate or inactivate a particular protein. Protein kinases may be classified by their substrate specificity. Eukaryotes have different kinases that phosphorylate Ser/Thr or Tyr but all eukaryotic kinases typically will fold into a similar active site with an activation loop and catalytic loop between which substrates bind.

Phosphatases may be grouped into phospho-Tyr phosphatases, phospho-Ser/Thr phosphatases and those that cleave both. Unlike kinases which differ in their structure of their catalytic domains, many phosphatases gain specificity by binding protein co-factors which facilitate the translocation and binding to specific phospho-proteins. The active phosphatase hence often consists of a complex of the catalytic subunit and a regulatory subunit. Some examples of important phosphatases include protein phosphatase 1 (PP-1), protein phosphatase 2A/B/C and protein Tyr-phosphatases 1B. Hence, the phosphorylation status of a protein is often determined by the delicate balance of kinases and phosphatases (108,109). The interplay between kinases and phosphatases turns 'on' or 'off' pathways within cells, thereby playing an important role in many intracellular signaling pathways (108).

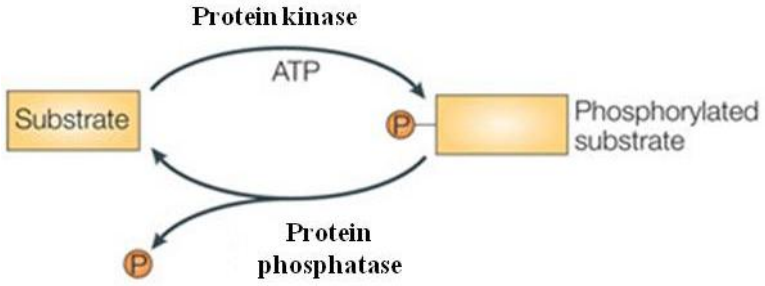

Figure 1.9 Schematic illustration of phosphorylation and dephosphorylation of a protein. Proteins are phosphorylated by kinases in an ATP-dependent manner while the removal of a phosphate group is catalyzed by protein phosphatases. Figure is modified and adapted from (110).

1.3.2.2 MAPK and PI3K signaling cascades

The activation of kinases by extracellular signals in signaling cascades such as the mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling cascades have been extensively studied. The MAPK

cascades are evolutionarily conserved, ubiquitous signaling modules that couple receptor-mediated events at the cell surface to cytosolic and nuclear effectors, regulating many basic cellular processes including, but not limited to, apoptosis, proliferation, differentiation and migration (111,112). All MAPK signaling cascades use a similar signal-relay mechanism that involves the sequential phosphorylation and activation of three kinases: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAP kinase (MAPK) (Fig 1.10). MAPKs are activated by dual phosphorylation on a conserved Thr-xxx-Tyr motif in their activation loop by upstream MAPKKs which are activated by dual phosphorylation by MAPKKKs (113). There are three major families of MAPK: the extracellular signal regulated protein kinase (ERK1/2); the c-Jun NH2 terminal kinase (JNK); and the p38 MAPKs, all of which respond to differing extracellular stimuli, resulting in a myriad of outcomes for cellular growth and proliferation. A handful of other MAPKs including ERK3 and ERK5 have been discovered but are less well understood (113). Due to the complex nature of these signaling pathways, only the activation of the Ras-Raf-MEK-ERK MAPK pathway will be discussed (Fig 1.11).

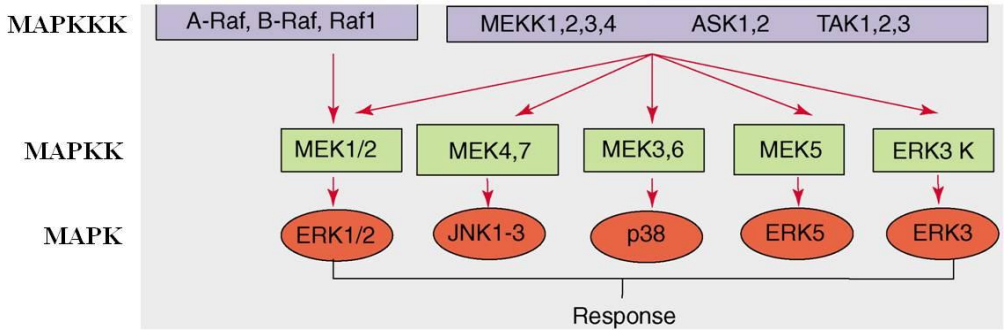

Figure 1.10 Schematic representation of signaling through classical MAPK cascades. Activation of the MAPKKK activates a MAPKK and the successive activation of the corresponding MAPK. Figure is modified and adapted from (113).

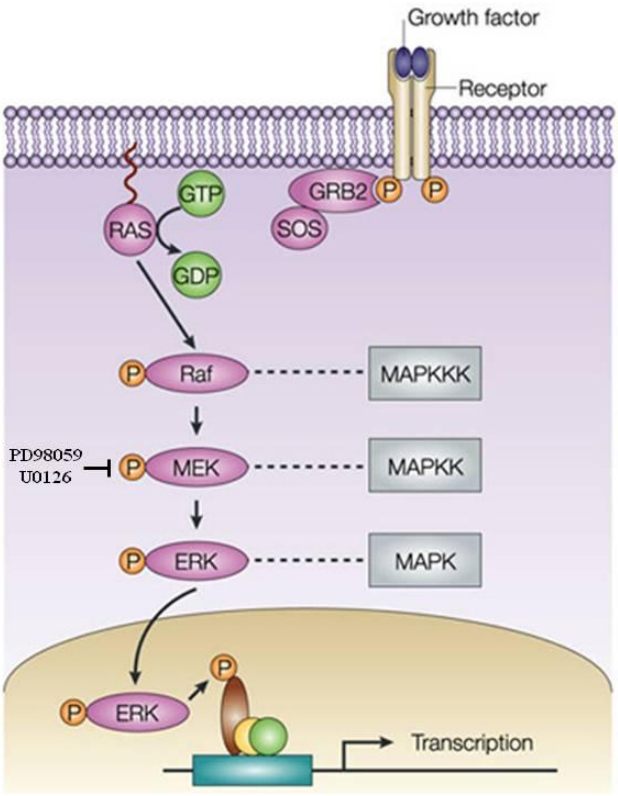

Figure 1.11 Activation of the RAS-Raf-MEK-ERK MAPK signaling cascade. Activation of the pathway occurs when extracellular ligands such as chemokines, growth factors and hormones bind to the cytokine receptors, receptor tyrosine kinase (RTKs) and G-protein couple receptors (GPCRs) respectively (114-116). Ligand binding to the extracellular domain of the receptors triggers the activation of the kinase in the cytosolic domain. This activation results in the phosphorylation of the C-terminal tyrosine residues that allows docking of the Src homology 2 (SH2) domain in adaptor proteins such as the growth-factor-receptor bound protein-2 (GRB2) (116). Adaptor protein GRB2 then recruits effector proteins such as guanine-nucleotide exchange factor son-of-sevenless (SOS) to the plasma membrane promoting the exchange of GDP for GTP on GTPase RAS, thereby activating RAS which is anchored at the cytosolic side of the plasma membrane. Activated RAS then recruits the inactive serine/threonine Raf (MAPKKK) from the cytosol to the plasma membrane and activates it (117,118). Subsequently, activated Raf phosphorylates and activates MEK1/2 (MAPKK), a dual specificity tyrosine and serine/threonine kinase which in turn catalyze the activation of serine/threonine kinase ERK1/2 (MAPK). Activated ERK1/2 subsequently phosphorylates transcription factors and cytosolic proteins such as p90 ribosomal S6 kinase (Rsk) (119-121). Figure is modified and adapted from (122).

The PI3Ks belong to a conserved family of lipid kinases that phosphorylates the 3'-hydroxyl group of phosphoinositides. Similar to the MAPK signaling pathway, the PI3K cascade responds to a variety of extra- and intracellular signals that regulates cellular proliferation, cell death and cytoskeletal rearrangements. In the recent years, the PI3K signaling pathway has been widely believed to play a major role in cancer and is recognized as one of the most frequently targeted signaling pathways in most sporadic tumours in humans (116,123). Interestingly, only class I PI3Ks are involved in cancer; there are to date, no data linking class II PI3Ks or class III PI3K to oncogenesis (124). This finding may reflect the different product and substrate specificities of the three classes of PI3K. Notably, only class I PI3Ks uses phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate phosphatidylinositol 3,4,5-triphosphate (PIP_3), class II PI3Ks produce the 3,4-bisphosphate and the 3-monophosphate of inositol lipids and class III can only make the 3-monophosphate (124). The activation and overall effect of the PI3K signaling, as illustrated in Fig 1.12, enhances the stimulation of cellular replication and survival and to reduce growth inhibition and apoptosis (124).

Notably, phosphorylation is one of the main mechanisms that directly regulate the protein stability and function of many pro-apoptotic and anti-apoptotic members of the Bcl-2 family (69). For instance, it was reported that AKT, a serine/threonine protein kinase downstream of PI3K, binds to and phosphorylates Bad at serine 136 residue (125,126). Upon phosphorylation, Bad dissociates from anti-apoptotic Bcl-xL and is bound and sequestered in the cytosol by 14-3-3 proteins thereby suppressing apoptosis and promoting cell survival (127). In another study, it was reported that anti-apoptotic protein,

Mcl-1 is phosphorylated by glycogen synthase kinase-3 (GSK-3) another key down-stream kinase of PI3K, at serine 159 residue (128). Notably, phosphorylation of Mcl-1 results in its poly-ubiquitination and the consequent degradation via the UPS (128). Similarly, it was demonstrated that ERK1/2 phosphorylation of Bcl-2 at serine 70 serine stabilizes Bcl-2:Bax interaction, thereby mediating cell survival by quenching the pro-apoptotic function of Bax (129).

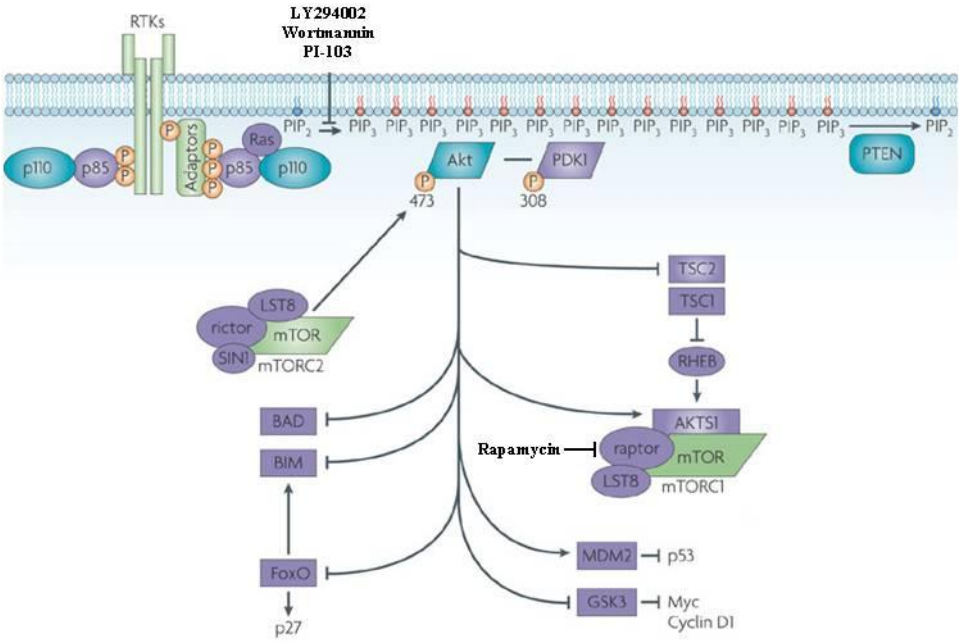

Figure 1.12 Schematic representation of PI3K signaling cascade. PI3Ks are heterodimers that comprise of catalytic (p110) and regulatory (p85) subunits. Following ligands binding at the extracellular receptors, the phosphorylated receptors sequester the regulatory subunit of PI3Ks, freeing the catalytic subunit to catalyze the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-triphosphate (PIP_3) (130). PIP_3 is the main propagator for the PI3K cascade and its production is mostly deterred by phosphatase and tensin homolog (PTEN) which hydrolyzes PIP_3 to PIP_2, terminating the PI3K signaling (131). Tumor suppressor PTEN has been shown to be frequently inactivated or silenced in many caners. PIP_3 then binds to pleckstrin homology (PH) domains of proteins such as phosphoinositide-dependent kinase 1 (PDK1) resulting in the membrane localization of PDK1 (130,131). PDK1 then recruits serine-threonine kinase AKT to the membrane. Phosphorylation and consequent activation of AKT then results in the activation of a range of downstream targets in the nucleus and cytosol (130,131). Signals originating from AKT proceeds through the tuberous sclerosis complex (TSC), Rheb (Ras homolog enriched in brain) and mTOR (target of rapamycin) ultimately, resulting in increased cellular growth and survival. Figure is modified and adapted from (132).

1.3.3 Acetylation

1.3.3.1 Protein acetylation, a relatively new protein regulatory mechanism

Protein acetylation is a reversible and highly regulated PTM first discovered on histones in 1968 (133) but the enzymes responsible for the acetyl group addition to or removal from target proteins known as histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively were not identified until 1995 (134). In the past decade, tremendous work investigated on protein acetylation has led to better understanding of this PTM, with targets rapidly expanding from histones to transcription factors, metabolic enzymes and signaling regulators in the cytosol (135). Notably, protein acetylation is now believed to be one of the most common covalent modifications of eukaryotic proteins (136). In fact, it has been argued that the frequency and importance of acetylation might actually rival phosphorylation as a crucial post-translational modification with as many as 50%-80% of proteins reportedly modified with acetylation (106,136,137). Moreover, comparative analyses showed that acetylation sites are significantly more conserved than phosphorylation sites (138). Since lysine acetylation results in the loss of positive charge at lysines, it strongly modulates the electrostatic properties (139). As such, the functional consequences may vary with the relative position of specific lysine residues within the protein and may influence the subcellular localization, activity, transcriptional activity, protein stability and protein-protein interaction of the targeted protein (137,139-142).

Notably, among these cellular functions, the regulation of protein stability by acetylation has emerged as a general phenomenon involving

different functional groups of proteins (143). The fundamental relationship between protein acetylation and ubiquitination is the nature that both PTMs target lysine residues on proteins (143). Besides acetylation and ubiquitination, lysines are the target of additional PTMs, including sumoylation and methylation which may all compete with each other for one targeted lysine. A prior lysine acetylation could affect subsequent protein ubiquitination and vice versa. In fact, two main mechanisms involving the regulation of protein stability following lysine acetylation have been proposed (Fig 1.13) (143). Hence, similar to phosphorylation, protein acetylation may serve as an important regulator of various cellular functions including protein ubiquitination.

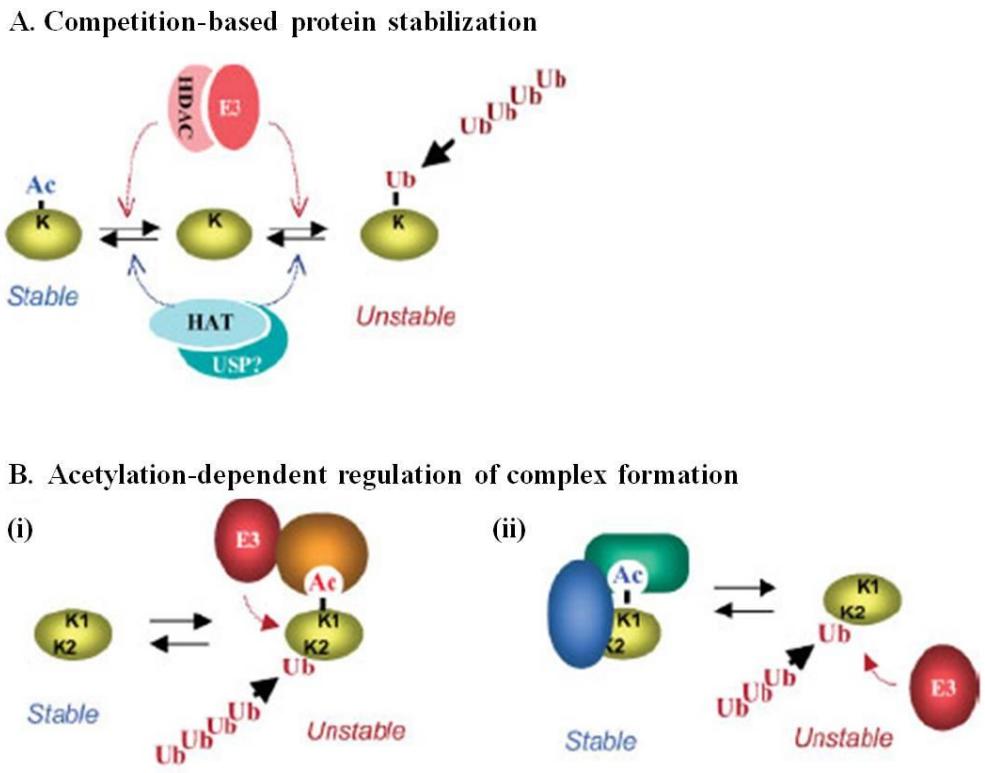

Figure 1.13 Two main proposed mechanisms that may regulate protein stability following lysine acetylation on protein residues. **(A)** Competition-based protein stability. Since lysines are sites of both ubiquitination and acetylation, acetylation of lysine residues may block their ubiquitination and degradation, leading to the stabilization of the protein. Conversely, deacetylases (HDACs) would remove the acetyl moiety, freeing the lysine residue for ubiquitination and the subsequent

degradation by the UPS. Similarly, acetylases (HAT) and deubiquitinases (USP) would ensure the stability of the de-ubiquitinated proteins by protecting the target lysines from the E3 ubiquitin ligases. **(B) (i)** Acetylation of a specific lysine residue may create a binding site for the recruitment of an E3 ubiquitin ligase resulting in its poly-ubiquitination and degradation of the protein. **(ii)** Conversely, an acetylated lysine may mask the targeted lysine residues, thereby protecting them from being ubiquitinated. Figure is modified and adapted from (143) .

1.3.3.2 Sirtuin family of deacetylases

Present knowledge in this field suggests that the fine balance between HATs and HDACs provides a key contribution to the modulation of protein acetylation and hence the regulation of many cellular functions (135). HATs are grouped into two main families, HAT-A and HAT-B, depending on the mechanism of catalytic action and on cellular localization (135). HAT-A family of proteins such as HIV type 1-interacting protein (TIP60) are localized in the nucleus where they transfer the acetyl group from acetyl-coenzyme A (acetyl-CoA) to an epsilon-amino group of histone after the assembly into nucleosomes. On contrary, members of HAT-B family act in the cytosol and transfer the acetyl group from acetyl-CoA to an epsilon-amino group of free histones prior to their deposition on the DNA (135). Conversely, HDACs can be classified into four main groups in relation to their phylogenetic conservation (144). While classes I, II and IV encompass the classical family of zinc-dependent HDACs, class III comprises of the nicotinamide adenine dinucleotide (NAD^+)-dependent sirtuin family of proteins (145,146).

Founded by the yeast Sir2 (147), the sirtuin protein family represents a promising new class of protein deacetylases which have been linked to life-span regulation, cell survival, apoptosis, gene expression, DNA repair and metabolic process regulation (146). In humans, seven sirtuins isoforms,

SIRT1-7 have been identified which share a common catalytic core domain comprising approximately 200-275 amino acids but also possess distinct N- and C- terminal extensions (Fig 1.14). The catalytic core domain elicits NAD^+-dependent deacetylase and/or ADP-ribosyl transferase, demalonylase and desuccinylase activities, giving each sirtuin a characteristic enzymatic activity (148). With the exception of SIRT4, the sirtuin family proteins hydrolyze one NAD^+ for each acetyl group removed from the substrate, along with the release of the nicotinamide moiety (149). The acetyl group is transferred to ADP-ribose to form a novel *O*-acetyl-ADP-ribose product (150) which has been proposed to serve as a secondary messenger (151). Notably, SIRT4 is the only sirtuin member that exhibits no histone deacetylase activity (152). In addition, it was revealed that SIRT5 is also capable of removing acyl-groups from malonylated or succinylated substrate peptides, thereby forming *O*-malonyl-ADP-ribose or *O*-succinyl-ADP-ribose respectively (153,154). Furthermore, SIRT4 and SIRT6 were shown to catalyze the transfer of ADP-ribose from NAD^+ to substrate proteins (152,155). These findings provide evidence that sirtuins may be able to function in more than one biochemical reaction.

In addition to their distinct enzymatic activities, the seven mammalian sirtuins are also localized in different sub-cellular compartments. Being localized in the nucleus, SIRT1 deacetylates several lysine residues of histones, including acetylated lysines 9 of histones H3 (H3K9ac), H3K14ac, H4K16ac and H1K26ac (156,157). SIRT1 also targets and deacetylates non-histone proteins and its activity may be influenced by its ability to shuttle between nuclear and cytosolic compartments (158,159). For instance, SIRT1 also

deacetylates and activates the transcriptional co-activator PGC-1α, a key regulator of mitochondria biogenesis (160). Over the years, it is becoming apparent that SIRT1 is intimately involved in the regulation of a wide range of cellular processes such as lipid and glucose metabolism, aging and stress response. Of which, the AMP-activated protein kinase (AMPK)-SIRT1 signaling axis is of particular interest. AMPK functions as an energy sensor that is activated in response to cellular metabolic stress including calorie restriction (161). As such, it is not surprising then that *SIRT1* knockout mice are found to have a high prenatal or early post-natal death rate (162,163).

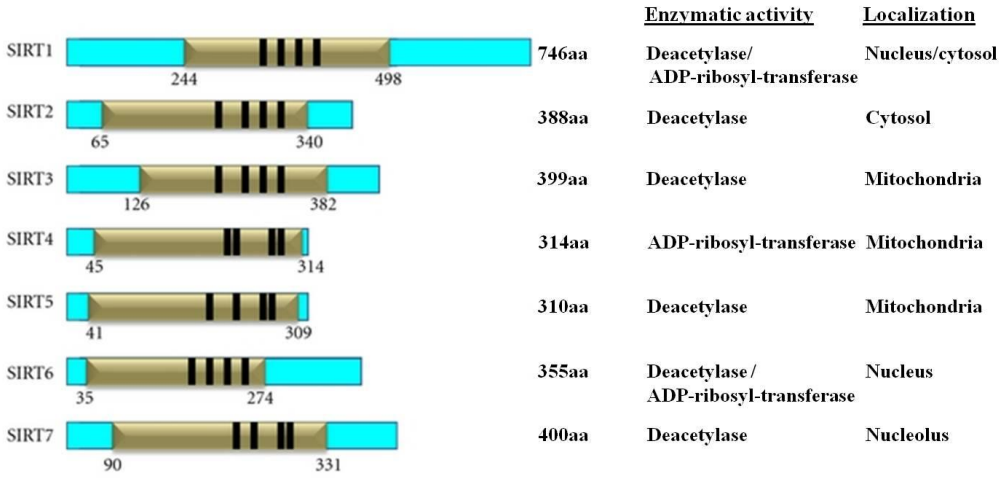

Figure 1.14 Schematic overview of human sirtuins, their enzymatic activity and sub-cellular localization. The seven mammalian sirtuins share a conserved catalytic domain of 200-275 amino acids (aa) and possess zinc-binding domain (indicated in gold and black respectively). In contrast to the conserved catalytic domain, the sirtuins differ in sequence and length of their C- and N-terminal extensions. Figure is modified and adapted from (164).

SIRT2 is the only human sirtuin which is pre-dominantly localized in the cytosol, but it may also be present in the nucleus where it selectively deacetylate histone H4K16 (165). Notably, human SIRT2 is expressed in two isoforms; variant 1 comprises of 389 amino acids while variant 2 does not

have the first 37 N-terminal amino acids and hence is consists of 352 amino acids (166). In the cytoplasm, SIRT2 shares α-tubulin deacetylase activity with histone deacetylase 6 (HDAC6) and has been proposed as the key microtubule deacetylase in neurons (167). Moreover, SIRT2 binds to 14-3-3 which is involved in controlling a variety of signaling molecules such as kinases and phosphatases (168). The SIRT2:14-3-3 interaction enhances AKT-dependent deacetylation of p53 by SIRT2, thereby down-regulating the transcriptional activity of p53 (168). These findings suggest a novel regulation of p53 mediated by SIRT2 alongside the well-characterized Mdm2-mediated regulation.

SIRT3-5 are also referred to as 'mitochondrial sirtuins' due to their exclusive subcellular localization. SIRT3 is the primary deacetylase in the mitochondria where it regulates oxidative phosphorylation, protein synthesis and the cellular response to oxidative stress and calorie restriction (156). Indeed, *SIRT3* knockout mice showed increased susceptibility to oxidative stress and metabolic damage after high fat feed (169). SIRT4 resides as a soluble protein in the mitochondrial matrix (152). While SIRT4 exhibits no histone deacetylase activity, it functions as an efficient ADP-ribosyltransferase (152). Notably, it was shown that SIRT4 ADP-ribosylates and deactivates glutamate dehydrogenase (GDH) which is required to promote the metabolism of glutamate and glutamine to generate ATP, thereby promoting the secretion of insulin (170). Knockdown of SIRT4 in insulinoma cells activates GDH, leading to increased amino-acid-stimulated insulin secretion (170). Moreover, GDH from SIRT4 knockout mice is resistant to phosphodiesterase, an enzyme that cleaves ADP-ribose, suggesting the absence of ADP-ribosylation on GDH

(170). These findings demonstrate that SIRT4 plays a role in beta cell mitochondria to suppress the activity of GDH by ADP-ribosylation, thereby repressing amino acid-stimulated insulin secretion. In addition to its pronounced demalonylase and desuccinylase activity (171), SIRT5 was shown to deacetylate and activate carbamoyl phosphate synthetase 1 (CPS1), an enzyme which catalyzes the initial step of the urea cycle for ammonia detoxification and disposal (172). Indeed, *SIRT5* knockout mice failed to up-regulate the activity of CPS1 during fasting (172). These results indicate that SIRT5 plays an important role in ammonia detoxification and disposal by deacetylating and activating CPS1.

SIRT6 is a nuclear, chromatin-associated protein which promotes resistance to DNA damage and represses genomic instability in mouse cells (173,174). In comparison to the other sirtuins knockout mice, *SIRT6* knockout mice displayed a severe phenotype. *SIRT6* knockout mice are small and at 2-3 weeks of age develop severe metabolic defects (174). Eventually, these mice will die at four weeks of age due to degenerative processes of multiple organs (174). In addition, the importance of SIRT6 for telomere maintenance and genome stabilization has also been documented (175). These findings demonstrate the importance of SIRT6 in DNA repair and thus in the maintenance of genomic integrity. Out of the seven human sirtuins, SIRT7 is thus far the least studied. It is a nuclear protein that is predominantly localized in the nucleoli where it associates with and up-regulates the rDNA transcription (176-179). Knockdown of SIRT7 in human cancer cell-lines suppresses cell proliferation and induces apoptosis, suggesting that SIRT7 may be required for cancer cell viability (177). In addition, SIRT7 was found

to deacetylate and inactivate p53 (180). Notably, *SIRT7* knockout mice are viable but suffer from progressive heart hypertrophy, accompanied by inflammation and decreased stress resistance, possibly a consequence of altered p53 activity.

1.3.4 PTM regulation of MOAP-1

Characterization study done by our laboratory has shown that MOAP-1 is a short-lived protein with a half-life of approximately 25 minutes and is constitutively degraded by the UPS under non-apoptotic conditions (67). Interestingly, multiple apoptotic stimuli stabilize MOAP-1 through inhibition of its ubiquitination-mediated degradation process (67). Furthermore, it was showed that post-translational mechanism instead of transcriptional or translational mechanisms plays a significant role in mediating the stabilization of MOAP-1 under apoptotic conditions (67). TRIM39, a member of the RBCC/TRIM family (briefly discussed in Section 1.3.1) was the first regulator of MOAP-1 protein stability identified. TRIM39 was found to interact with and stabilize MOAP-1 by inhibiting its poly-ubiquitination process thereby reducing the degradation of MOAP-1 by the UPS (103). In agreement with its effect on enhancing MOAP-1 protein stability, TRIM39 sensitizes cells to ETOP-induced apoptosis while the loss of function analysis showed that knockdown of TRIM39 reduces the sensitivity of the cells to ETOP-stimulated apoptosis (103). In addition, elevation of MOAP-1 protein levels by over-expression of TRIM39 promotes Bax-mediated cytochrome c release in isolated mitochondria (103). Three years later in 2012, it was revealed by another group that MOAP-1 is a novel substrate of anaphase-promoting

complex (APC/C^{Cdh1}) E3 ubiquitin ligase and that TRIM39 stabilized MOAP-1 by inhibiting APC/C^{Cdh1} and consequently the APC/C^{Cdh1}-mediated ubiquitination of MOAP-1 which prevents the degradation of MOAP-1 by the UPS (104). However, the precise mechanism of the inhibition of APC/C^{Cdh1} by TRIM39 is still unknown.

Thus far, the only PTM regulation of MOAP-1 identified is ubiquitination whereby poly-ubiquitination of MOAP-1 will result in its degradation by the UPS. Apoptotic stimuli and over-expression of TRIM39 have been showed to inhibit the poly-ubiquitination of MOAP-1 thereby reducing its degradation by the UPS. However, it is highly conceivable that the molecular regulation of MOAP-1 is linked to a highly orchestrated cascade of ubiquitination/de-ubiquitination events that are intermingled with phosphorylation/de-phosphorylation, acetylation/deacetylation and other post-translational modification events.

1.4 OBJECTIVES OF STUDY

Bax has been shown to be a critical and essential effector for the mitochondrial-mediated apoptosis pathway (62-64). MOAP-1 was cloned as a binding partner of Bax and knockdown analysis of MOAP-1 has been shown to deter proper functioning of Bax thereby resulting in dramatic resistance of cancer cells to multiple apoptotic stimuli (65,66). Since levels of MOAP-1 has been demonstrate to modulate the sensitivity of cancer cells to Bax-mediated apoptosis, the investigation on the molecular regulation of MOAP-1 may lead to the identification of potential targets to elevate levels of MOAP-1 in cancer cells, thereby ensuring optimal Bax functioning and consequently increasing the sensitivity of cancer cells to chemo-therapeutic treatments. Thus, the doctoral research aims to investigate post-translational regulatory mechanism of MOAP-1.

To achieve the aim, the following objectives are set:

1. To evaluate close homologues of TRIM39 that may potentially act as E3 ubiquitin ligase of MOAP-1

2. To investigate the role of phosphorylation as a possible mechanism for regulating the protein stability and function of MOAP-1

3. To evaluate sirtuin family of deacetylases as novel interactors and regulators of MOAP-1

The results from this investigation are presented in Chapters 3, 4 and 5 respectively.

CHAPTER 2

Materials and Methods

2.1. MATERIALS

Materials and antibodies with their suppliers are enlisted in Table 2.1 and Table 2.2 respectively. Plasmids used in this study were enlisted in Table 2.3.

Table 2.1 List of materials

Material	Supplier
Acetone	Merck
Acrylamide/bis-acrylamide 30% solution 37:1:1	Bio-Rad
Agarose	Invitrogen
Ammonium persulfate (APS)	Sigma Aldrich
Ampicillin	Sigma Aldrich
Adenosine 5'-triphosphate (ATP)	Sigma Aldrich
β-mercaptoethanol	Bio-Rad
Bovine serum albumin	Sigma Aldrich
Bradford dye reagent	Bio-Rad
Bromophenol blue	Sigma Aldrich
CL-Xposure® X-ray films	Thermo Scientific
Commassie Brillant blue R-250	Bio-Rad
Cycloheximide	Sigma Aldrich
Dimethylsulfoxide (DMSO)	Sigma Aldrich
DJNK1 (JNK inhibitor)	Enzo Life Sciences
DMEM medium (high glucose)	Sigma Aldrich
DpnI	New England Biolabs
Ethanol	Merck
Ethylenediamine tetracetic acid (EDTA)	Sigma Aldrich
Etoposide	Sigma Aldrich
Fetal bovine serum (FBS)	Sigma Aldrich
Glutathione-Superflow™ Resin	Clontech
Glycerol	Invitrogen
Glycine	Bio-Rad
HEPES	Sigma Aldrich
HotStarTaq *Plus* DNA polymerase	Qiagen
LB Broth	Sigma Aldrich
L-glutamine	GIBCO
Lipofectamine 2000	Invitrogen
Isopropyl beta-thiogalactoside (IPTG)	Invitrogen
LY294002 (PI3K inhibitor)	Sigma Aldrich
Methanol	Fisher Scientific

Material	Supplier
Nitrocellulose membrane	Bio-Rad
Nonidet P-40 (NP-40)	Sigma
PD98059 (MEK inhibitor)	Calbiochem
Penicillin-Streptomycin	Sigma Aldrich
Pfu DNA polymerase	Promega
Phenylmethylsulfonyl fluoride (PMSF)	Sigma Aldrich
Phosphate buffered saline	1st Base
PhosSTOP Phosphatase Inhibitor Cocktail Tablets	Roche Diagnostics
PI-103 (dual inhibitor of PI3K and mTOR)	Cayman Chemical
Polyvinylidene difluoride membrane (PVDF)	PerkinElmer
Ponceau S	Sigma Aldrich
Precision Plus Protein™ All Blue Standards	Bio-Rad
Protease inhibitor cocktail tablets	Roche Diagnostics
Protein A Sepharose beads	Roche Diagnostics
Qiaprep miniprep spin kit	Qiagen
Q Sepharose Fast Flow	GE Healthcare
Rapamycin (mTOR inhibitor)	Santa Cruz
Reduced glutathione	Sigma Aldrich
Restore Western Blot Stripping Buffer	Thermo Scientific
SB203580 (p38 inhibitor)	Cell Signaling
Sodium bicarbonate	AnalaR Normapur
Sodium chloride	Sigma Aldrich
Sodium dodecyl sulfate (SDS)	Merck
Sodium hydroxide	Merck
Sodium pyruvate	GIBCO
SP Sepharose Fast Flow	GE Healthcare
SuperSignal West Pico Chemiluminescent Substrate	Thermo Scientific
SYBR Safe DNA gel stain	Molecular Probe
N,N,N',N'-tetramethylene ethyldiamine (TEMED)	Sigma Aldrich
TNF-related apoptosis-inducing ligand (TRAIL)	PeproTech Inc.
Tris	Sigma Aldrich
Tris-acetate-EDTA (TAE)	1st Base
Triton X-100	BDH
Trypsin/EDTA (1x)	GIBCO
Tween 20	Sigma Aldrich
U0126 (MEK inhibitor)	Promega
X-ray developer	Konica Minolta
X-ray fixer	Konica Minolta
Wortmannin (PI3K inhibitor)	Sigma Aldrich
Z-Leu-Leu-Leu-al (MG132)	Sigma Aldrich

Table 2.2 List of antibodies

Primary Antibody	**Species**	**Supplier**	**Working Concentration**
Bax (2D2)	Mouse	Sigma-Aldrich	1:1000
Bax (N20)	Rabbit	Santa Cruz	1:1000
Cleaved Caspase 7	Rabbit	Cell Signaling	1:1000
ERK	Rabbit	Cell Signaling	1:1000
FLAG (M2)	Mouse	Sigma-Aldrich	1:5000
HA (F7)	Mouse	Santa Cruz	1:1000
HA (Y11)	Rabbit	Santa Cruz	1:1000
HSP60	Mouse	Sigma-Aldrich	1:5000
Mcl-1 (S19)	Rabbit	Santa Cruz	1:1000
MOAP-1	Rabbit	Sigma-Aldrich	1:1000
MYC (9E10)	Mouse	Santa Cruz	1:1000
MYC (A14)	Rabbit	Santa Cruz	1:1000
Phospho-ERK (Thr202/Tyr204)	Mouse	Cell Signaling	1:1000
SIRT1	Mouse	Sigma-Aldrich	1:1000
SIRT2	Mouse	Sigma-Aldrich	1:1000
Ubiquitin (P4D1)	Mouse	Santa Cruz	1:1000
Secondary Antibody	**Species**	**Supplier**	**Working concentration**
Horse radish peroxidase	Mouse	GE Healthcare	1:5000
Horse radish peroxidase	Rabbit	GE Healthcare	1:5000
Cyanine 3	Mouse	Invitrogen	1:500

Table 2.3 List of plasmids

Plasmids used in Chapter 3	**Expression**
PXJ-HA-PNMA1	Mammalian
PXJ-HA-PNMA2	Mammalian
PXJ-HA-PNMA3	Mammalian
PXJ-HA-PNMA4/ HA-MOAP-1	Mammalian
PXJ-HA-PNMA 5	Mammalian
PXJ-HA-PNMA6	Mammalian
PXJ-HA-UBC8	Mammalian
PXJ-HA-UBC13	Mammalian
pCMV6-MYC-FLAG-TRIM11 WT	Mammalian
pCMV6-MYC-FLAG-TRIM11 C16,19A,	Mammalian
pCMV6-MYC-FLAG-TRIM11 C31A, H33A	Mammalian
pCMV6-MYC-FLAG-TRIM21	Mammalian
pCMV6-MYC-FLAG-TRIM39	Mammalian
pGEX-4T1-FLAG-MOAP-1	Bacterial
pGEX-4T1-TRIM11 WT	Bacterial
pGEX-4T1-TRIM11 C16,19A	Bacterial
pGEX-4T1-TRIM11 C31A,H33A	Bacterial
Plasmids used in Chapter 4	**Expression**
PXJ-FLAG-MOAP-1	Mammalian
PXJ-HA-MOAP-1 WT	Mammalian
PXJ-HA-MOAP-1 S27,29,31A	Mammalian
PXJ-HA-MOAP-1 S27,29,31D	Mammalian
PXJ-HA-MOAP-1 S169,173A	Mammalian
PXJ-HA-MOAP-1 S169,173D	Mammalian
PXJ-HA-MOAP-1 S27,29,31,169,173A	Mammalian
PXJ-HA-MOAP-1 S27,29,31,169,173D	Mammalian
PXJ-HA-ERK	Mammalian
PXJ-HA-MEKΔ	Mammalian
PXJ-MYC-MOAP-1	Mammalian
Plasmids used in Chapter 5	**Expression**
PXJ-FLAG-SIRT1	Mammalian
PXJ-FLAG-SIRT2	Mammalian
PXJ-FLAG-SIRT3	Mammalian
PXJ-FLAG-SIRT4	Mammalian
PXJ-FLAG-SIRT5	Mammalian
PXJ-GFP-MOAP-1	Mammalian

2.2 MAMMALIAN CELL CULTURE AND DRUG TREATMENT

2.2.1 General cell culture and maintenance

Cell culture procedures were performed in a class II biological safety cabinet. HEK293T (human embryonic kidney cell-line) and HeLa (human cervical adenocarcinoma cell-line) cells were maintained in DMEM medium containing 4500 mg/L glucose, 3.7 g/L sodium carbonate, 100 mg/L sodium pyruvate, 100 units/ml penicillin, 1 μg/ml streptomycin and 10% v/v heat-inactivated fetal bovin serum (FBS). For heat inactivation, FBS was incubated in a water bath at 55 ^{o}C for 1 h. Cells were sub-cultured once they were 80-90% confluent. Both HEK293T and HeLa cells were cultured in 100-mm plates. For sub-culturing, spent culture medium in the plate was aspirated and the cell mono-layer was rinsed with 1x PBS solution. To detach cells from the plate surface, 1 ml of Trypsin/EDTA 1x solution was applied onto the cell monolayer and the plate was incubated at 37 ^{o}C for 1-2 min until the cells had detached. Cells were re-suspended in fresh medium and sub-cultured in ratio ranging from 1:3 to 1:6 into new 100-mm plates. All cells were maintained at 37 ℃ in a humidified atmosphere of 95% air and 5% CO_2 with CO_2 incubator (Thermo Forma Series 2). For cell maintenance, cells were not cultured for more than 20 passages to minimize phenotypic alterations.

2.2.2. Cell freezing and thawing

To detach cells from the plate surface, the medium was aspirated and the cell monolayer was rinsed with 1x PBS solution. 1 ml of Trypsin/EDTA 1x solution was applied onto the cell monolayer and the plate was incubated at 37 ^{o}C for 1-2 min until the cells had detached. Trypsinization was stopped by

addition of DMEM containing 10% FBS and penicillin streptomycin. Cells were centrifuged at 1,200 rpm for 5 min. The supernatant was aspirated and the cell pellet was re-suspended in 500 µl of pre-sterilized (using a 0.20 µm filter] cell freezing medium containing 10% DMSO, 50 % FBS and 40 % DMEM. Cells were transferred to a Nalgene® cryogenic vial (CORNING) and frozen at -80 ℃. For long-term storage, the cryogenic vial was transferred into a liquid nitrogen storage tank. To thaw cells, the vial was incubated at 37 ℃ for 1 min, and the cell resuspension was added to 2 ml of DMEM containing 10 % FBS and penicillin streptomycin, followed by centrifugation at 1,200 rpm for 5 min. The supernatant was discarded, and the cell pellet was resuspended in 10 ml of culture medium and plated in a 100-mm culture dish.

2.2.3 Preparation of drug stocks and usage of drugs

All compounds used were weighed accurately and dissolved in DMSO or water to prepare the appropriate stock solutions. Drug stocks were aliquoted into 20 µl per tube and stored at -20 ^{o}C for usage. Proteasome inhibitor MG132 was used at a final concentration of 10 µM while DNA-damaging agent, Etoposide (ETOP) was used at a final concentration of 100 µM. TNF-related apoptosis-inducing ligand (TRAIL) was used at concentrations ranging from 25 to 100 ng/ml. 100 µg/ml cycloheximide was used to block new protein synthesis in HEK293T.

For the kinase inhibitors treatment, HEK293T cells were seeded in 6-well plates approximately 24 h before treatment and cells were allowed to grow until 70-80% confluency. To study the effect of the kinase inhibitors on the up-regulation of MOAP-1 by ETOP, cells were pre-treated with various

kinase inhibitors and then treated with 100 μM ETOP for 16 h followed by analysis by western blotting. Kinase inhibitors were added to cells at doses and times specified in the figure legends.

2.2.4 Transient transfection

Cells were seeded in appropriate dishes approximately 24 h before transfection. Cells were allowed to grow until 60-70% confluency. Lipofectamine 2000 was used as the transfection reagent at a ratio 1 μl Lipofectamine 2000: 1 μg of plasmid DNA. In two separate tubes, the appropriate volume of Lipofectamine 2000 and plasmid DNA was diluted in serum-free medium and incubated at room temperature for 5 min. The two solutions were mixed and incubated for another 20 min at room temperature to allow the formation of DNA-liposome complexes. The transfection mixture was then added drop-wise into the culture medium. 6 h after the transfection, culture medium containing the transfection mixture was removed and the cells were incubated in complete growth medium for another 24-48 h before harvesting for western blotting analysis.

2.2.5 Transient knock-down of TRIM11 using siRNAs

The following GeneSolution siRNAs of human TRIM11 (catalogue number 1027416) was purchased from Qiagen,

	Product name	**Target sequence**
1.	Hs_TRIM11_1	AGAGTAGATGTCCTATAATAA
2.	Hs_TRIM11_2	AAGAATTAGGAGGCAGCCATA
3.	Hs_TRIM11_3	CAGGGAGAACGTGAACAGGAA
4.	Hs_TRIM11_4	CAGAGAGTAGATGTCCTATAA

100 µl of sterile RNase-free water was added to each tube containing 1 nmol lyophilized siRNA to obtain a stock solution of 10 µM solution and stored at -20 ^{o}C until transfection. Lipofectamine 2000 was used as the transfection reagent at a ratio 2 µl Lipofectamine 2000: 10 nM siRNA. siRNA transfection was carried as described in Section 2.2.3. The final working concentrations of siRNAs used were 10-20 nM and cells were harvested 72 h after transfection for analysis.

2.3 PROTEIN METHODOLOGY

2.3.1 Cell lysate preparation, immuno-precipitation and Bradford assay

Culture medium was aspirated and cells were rinsed in ice-cold 1x PBS solution containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. PBS was then aspirated. For direct western blotting analysis, cells were lyzed on ice in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.2, 1% deoxycholate, 1% Triton X-100, 2 mM EDTA pH 8.0, 0.1% SDS) supplemented with protease inhibitor cocktail for 30 min. For co-immuno-precipitation experiments, cells were lyzed in immuno-precipitation (IP) lysis buffer (250 mM NaCl, 50 mM HEPES, 5 mM EDTA, 1% Triton X-100, 1% NP40, pH 7.4) supplemented with protease inhibitor cocktail for 30 min on ice. While for immuno-precipitation of activated Bax, cells were lyzed in CHAPS lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% CHAPS) supplemented with protease inhibitor cocktail for 30 min on ice. Whole cell lysate was centrifuged at top speed 13,200 rpm for 10 min. After centrifuged,

the protein concentration in the supernatant was estimated by using the Bradford protein assay reagent.

In a 96-well plate, 2 µl of cell lysate was added to 198 µl of Bradford reagent and absorbance was measured at 595 nm using a UV/Visible spectrophotometer (Tecan). Readings were measured in triplicates for each sample and mean absorbance was determined. A standard plot was obtained for a range of BSA concentrations (0.1-2 µg/ml). Protein concentrations in cell lysates were then calculated based on the linear equation derived from the standard plot. Total cell lysates containing equal amount of proteins were rotated with appropriate antibodies (normally, 1-2 µg) for 4 hour at 4 ^{o}C, followed by incubation with Protein A agarose beads for 2 hour. The beads were washed with an appropriate buffer for at least 5 times. 20 µl of 5X SDS loading buffer (200 mM Tris-HCl, pH 6.8, 8% SDS, 4% β-mercaptoethanol, 40% glycerol, 50 mM EDTA, 0.08% Bromophenol blue) was added to the beads and heated at 100 ^{o}C for 5-10 min. Total cell lysates and precipitates were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3.2 SDS-PAGE gel electrophoresis

Proteins contained in the cell lysates were separated using SDS-PAGE. Resolving gels containing 10 or 13.5% w/v acrylamide and stacking gels containing 4% acrylamide were prepared by using the following formulae:

Table 2.4 Formulae for resolving and stacking gel solutions for 4 SDS-PAGE gels

Ingredient	**Resolving gel solution**		**4% Stacking gel solution**
	10%	**13.50%**	
dH_2O (mL)	12	8.5	6.1
Tris (mL)	7.5 (1.5 M, pH 8.8)	7.5 (1.5 M, pH 8.8)	2.5 (0.5 M, pH 6.8)
30% Acrylamide (mL)	10	13.5	1.3
10% SDS (µL)	300	300	100
10% APS (µL)	200	200	50
Temed (µL)	20	20	10

All reagents required for resolving gel were mixed and approximately 30 ml of the resolving gel solution was quickly poured into four casting cassettes. An overlay of methanol was applied onto the surface of the gel solution. The gel was allowed to polymerize for about 20 min after which the layer of methanol was discarded. Stacking gel solution was then poured over the resolving gel contained in the casting cassette. A 10- or 15-well comb was inserted immediately into the stacking gel solution to create wells for loading samples and allowed to polymerize for 20 min. For both resolving and stacking gels, APS and TEMED were added immediately before pouring the solution in the casting assembly to avoid premature polymerization. Once the stacking gel had polymerized, the comb was removed and the gel along with the casting cassette was placed in an electrophoresis tank containing running buffer. Proteins were separated at a constant voltage of 80 V for approximately 2 hour in a running buffer consisting of 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS. After separation, the proteins were transferred onto nitro-cellulose membranes under constant voltage of 100 V for approximately 2 hour in an ice-cold transfer buffer consisting of 25 mM Tris-HCl, 192 mM glycine and 20% v/v methanol. The proteins on the gel were

directly stained with Ponceau S and the membranes were washed with PBST (137 mM NaCl, 3 mM KCl, 25 mM Tris HCl pH 7.4 containing 0.1% v/v Tween 20) before proceeding with western blotting.

2.3.3 Western blotting

The transblotted membrane was blocked with 5% fat-free milk in PBST for 1 hour at room temperature followed by incubation with primary antibody for 2 hour at room temperature or up to overnight at 4 ℃ with gentle shaking. Membranes were washed thrice with PBST every 15 min before incubation with corresponding secondary antibody conjugated to HRP in 5% milk in PBST for 1 hour at room temperature. The membranes were washed thrice with PBST every 15 min to remove any unbound antibody. The transblotted proteins of interest were detected by applying equal volumes of reagent 1 and 2 of the ECL system (total volume 1-2 ml) to the membranes, capturing the emitted chemiluminescence onto CL-Xposure® X-ray films and developing the films using a Konica Minolta film developer.

2.3.4 Membrane stripping and re-probing

For antibody stripping, the nitrocellulose membrane was incubated with western stripping buffer (Thermo Scientific) for 45 min at room temperature with gentle shaking. Membranes were then blocked in 5 % milk/PBST for an hour before being probed with another primary antibody. Care was taken to ensure there was no signal carried over from the previous primary antibody.

2.3.5 Cycloheximide chase protein stability assay

HEK293T cells were seeded in 12-well plates approximately 24 h before treatment and cells were allowed to grow until 70-80% confluency. Culture medium was aspirated and replaced with fresh medium containing 100 µg/ml cycloheximide. Immediately after changing medium, the first set of cells were lyzed in RIPA lysis buffer supplemented with protease inhibitor cocktail. These samples were considered as 0 time point. This was repeated at 1, 3 and 5 hour time-point for over-expressed MOAP-1 and 0.5, 1 and 2 hour time-point for endogenous MOAP-1. Whole cell lysates were assessed by protein estimation assay in triplicates as in Section 2.3.1. 50 µg of total protein were analyzed by SDS-PAGE and western blotting as described in Sections 2.3.2 and 2.3.3. Mcl-1 was used as a positive control to ensure that the CHX assay worked as expected while HSP60 was used as an internal loading control.

2.3.6 Expression and purification of bacterial-expressed GST-FLAG-MOAP-1 recombinant protein

Plasmid encoding GST-FLAG-MOAP-1 protein was transformed into 100µl of *E. coli* BL21 (DE3) competent cells and the mixture was plated onto an ampicillin agar plate and incubated overnight at 37 ^{o}C. A single colony was picked and cultured in 10 ml Lysogeny broth (LB) containing 50 µg/ml ampicillin and incubated in a shaker at 37 ^{o}C to obtain saturated cultures. The saturated cultures were added to 400 ml of LB with 50 µg/ml ampicillin and grown for appropriate time to obtain 0.6-0.7 absorbance at 600 nm. The expression of recombinant proteins was induced overnight by 1 mM isopropyl

beta-thiogalactoside (IPTG) at 16 ℃. Bacteria were harvested by centrifugation at 5,000 rpm for 10 min at 4 ^{o}C. The bacteria pellet was rinsed once in 50 ml ice cold PBS and centrifuged at 5,000 rpm for another 10 min. The bacterial pellet was then re-suspended in 30 ml of ice cold PBS supplemented with protease inhibitors and lyzed by sonication or French Press. The lysates were then clarified by centrifugation at 250,000 rpm for 1 hour at 4 ^{o}C. The supernatant was collected and rotated with 500 µl of pre-washed glutathione–Sepharose beads for 1 hr at 4^{o}C. The beads were collected and washed several times with PBS. The GST-tagged protein was then eluted with 20 mM reduced glutathione in 50 mM Tris, pH 7.9. For the generation of recombinant FLAG-MOAP-1 without the GST tag, the beads were incubated with appropriate amount of thrombin for 1 hour at 4 ^{o}C. Thrombin was inactivated by the addition of protease inhibitor, PMSF. Eluted proteins were dialyzed using Slide-A-Lyzer® dialysis cassette in 50 mM Tris, pH 7.9 for overnight. The protein solution was concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (Millipore) following supplier's protocol. The final protein solution was stored at -80 ^{o}C until usage.

2.3.7 Indirect immuno-fluorescence

HeLa cells grown on cover-slips were fixed and permeabilized with methanol: acetone (1:1 ratio) for 30 min at 4℃. After sequentially washed with PBS, samples were then blocked with 1% w/v BSA in PBS for 30 min at room temperature. Cells were then incubated with anti-FLAG antibody (1:200 dilution) against protein of interest (FLAG-SIRT1-5) in 0.1% w/v BSA for 1 hour at room temperature with gentle shaking. After washing thrice with PBS for 15 minutes, cells were incubated with anti-mouse conjugated to Cy3

(1:500 dilution) for 1 hour at room temperature. Samples were washed thrice again at 15 min intervals to minimize non-specific binding. The plates were gently shaken during incubation to allow even distribution of the antibodies on the cover-slips. Immuno-labeled cover-slips were mounted on glass slides using 50% glycerol in water. Edges of the cover-slips were sealed onto the slide using clear nail polish. Images were obtained using confocal laser microscope (Olympus Fluoview FV10i Confocal Microscope, Japan).

2.3.8 *In vitro* phosphorylation assay

Recombinant GST-MOAP-1 was expressed and purified as described in Section 2.3.6. For the brain tissue harvest and preparation, the whole brain was taken out from a male mouse and homogenized in lysis buffer (25 mM HEPES pH 7.3, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 5% glycerol, 0.2% Triton X-100) supplemented with protease inhibitor in the ratio 1ml of lysis buffer to 0.1g of brain tissue. The tissue lysate was centrifuged for 100, 000g for 30 mins, 4 ^oC. The clarified tissue lysate was crudely purified using SP (cation exchanger) or Q (anion exchanger) Sepharose Fast Flow and eluted with 0.25M, 0.5M, 0.75M and 1M of NaCl. The protein concentrations of the eluates were determined with Bradford assay. For the collaboration with Jeff at IMCB, 5 μg purified recombinant GST-MOAP-1 was incubated with 4 μg of brain or liver lysates and 10μM [γ-^{32}P] ATP at 30 ^oC for 20 min in a kinase buffer (25 mM HEPES pH 7.3, 25 mM NaCl, 5 mM $MgCl_2$, 2.5 mM NaF, 5 mM β-glycerophosphate, 0.025% Triton X-100). The reactions were stopped by boiling the mixtures in 2x SDS-PAGE sample buffer, separated on SDS-PAGE, transferred to polyvinylidene

difluoride membrane (PerkinElmer Life Sciences), and auto-radiographed. All [γ-^{32}P] ATP radio-labeling experiments were conducted at a collaborator's laboratory in IMCB, Biopolis. For the mass spectrometry collaboration with Li Rong at ETC, similar *in vitro* phosphorylation reactions were conducted as described above. However, ATP was used in replacement of [γ-^{32}P] ATP. Phosphorylation reactions were stopped by boiling the mixtures in 2x SDS-PAGE sample buffer, separated on SDS-PAGE. The gels were then submitted to the collaborator for mass spectrometry analysis.

2.3.9 *In vitro* ubiquitination assay

The ubiquitination reaction was conducted by the Prof Wong Hong Rui's laboratory in Xiamen University. Briefly, the *in vitro* ubiquitination assay was conducted by incubating E1 activating enzyme, E2 conjugating enzyme (UbcH5C), GST-TRIM11 (as the E3 ubiquitin ligase), FLAG-MOAP-1 (as the substrate), adenosine 5'-triphosphate (ATP) and ubiquitin in a reaction buffer containing 50 mM Tris, 5 mM $MgCl_2$ and 2 mM DTT at pH 7.5. GST-TRIM11 and FLAG-MOAP-1 were expressed and purified by GST-pull down as described in Section 2.3.6. The reaction was incubated at 30 ^{o}C for 2 hour. For total lysates, samples were boiled in loading buffer to stop the reaction and subjected to SDS-PAGE and western blotting as described above. For the *in vitro* ubiquitination assay followed by co-immuno-precipitation assay, reaction samples were boiled in 1% SDS to terminate the reaction then diluted 10 times in 0.5% TNTE and precipitated with anti-FLAG antibody to pull down ubiquitinated-conjugated MOAP-1. Similar to total lysates, the precipitates were subjected to SDS-PAGE and western blotting as described above.

2.4 MOLECULAR BIOLOGY TECHNIQUES AND METHODS

2.4.1 Plasmid DNA transformation

Pure plasmid DNA (100-500 ng) was added into 100 µl of competent cells, which were pre-thawed on ice. The mixture was incubated on ice for 30 min, heat-shocked for 45 sec at 42 ℃ water bath and immediately put back on ice for another 2 min. 1 ml pre-warmed LB was added into the mixture, followed by shaking at 37 ℃ warm room for 1 hour. The cells were pelleted by centrifugation at 8,000 rpm for 1 min at room temperature. Most of the supernatant was discarded and the cells were re-suspended in the remaining supernatant and spread on the LB agar plates containing the appropriate antibiotic and incubated overnight at 37 ^{o}C.

2.4.2 Mini-preparation of plasmid DNA

The Wizard® *Plus* SV Minipreps DNA Purification System (Promega) was used for rapid isolation of small-scale plasmid DNA. The 2-4 ml overnight grown bacteria cultures were harvested by centrifugation for 5 min at 4,000 rpm and the supernatant was discarded. The pellet was completely re-suspended in Cell Re-suspension Solution (50 mM Tris-HCl, Ph 7.5, 10 mM EDTA and 100 µg/ml RNase A) by vortexing, followed by the addition of Cell Lysis Solution (0.2M NaOH and 1% SDS). After incubation of the mixture for 5 min at room temperature, Alkaline Protease Solution was added, followed by incubation for another five minutes. The bacterial lysate was centrifuged at 13,200 rpm for 10 min at room temperature immediately after the addition of Neutralization Solution (4.09M guanidine hydrochloride, 0.759M potassium acetate and 2.12M glacial acetic acid, pH 4.2). The cleared

lysate was transferred to the Spin column and centrifuged for 1 min at 13,200 rpm at room temperature. Column Washing Solution (162.8 mM potassium acetate, 22.6 mM Tris-HCl, Ph 7.5 and 0.109 mM EDTA, pH8.0) previously diluted with 95% ethanol was added to the Spin Column, followed by another centrifugation. The wash procedure was repeated once more before eluting DNA with 40-100 µl of distilled H_2O. Concentration of the plasmid DNA was determined using the NanoDrop 1000 spectrophotometer (Thermo Scientific). All plasmids were sent for sequencing to ensure no unwanted mutations were introduced.

2.4.3 Maxi-preparation of plasmid DNA

Large-scale plasmid DNA isolation was performed by using the QIAGEN Plasmid Maxi Kit according to the manufacturer's instructions. 400 ml of overnight grown bacteria cultures were harvested by centrifugation and subsequently incubated with P1 (Re-suspension Buffer), P2 (Lysis Buffer) and P3 (Neutralization Buffer), which were similar to the procedures of mini-prep as described above. After centrifugation, the clear supernatant was carefully transferred to the pre-equilibrated QIAGEN-tip 500 column, followed by washing with Buffer QC (1.0M NaCl, 50mM MOPS pH 7.0 and 15% isopropanol). The DNA was eluted by Buffer QF (1.25M NaCl, 50 mM Tris-HCl, pH 8.5 and 15% isopropanol) and precipitated by isopropanol. The DNA pellet was washed with 70% ethanol, air-dried and dissolved in 500-800 µl of distilled H_2O. Concentration of the plasmid DNA was determined using the NanoDrop 1000 spectrophotometer (Thermo Scientific). All plasmids were sent for sequencing to ensure no unwanted mutations were introduced.

2.4.4 Agarose gel electrophoresis

To pour a gel, agarose powder was mixed with TAE buffer (40 mM Tris-acetate and 2 mM EDTA) to the desired concentration. SYBR Safe DNA gel stain was added to the gel at this point to facilitate visualization of DNA after electrophoresis. Samples containing DNA mixed with loading buffer were pipetted into the sample wells. Agarose gel electrophoresis was carried out at constant voltage 100 V in TAE buffer. 100 bp or 1 kb DNA ladders (New England Biolabs) were used to determine the size of the DNA. The DNA bands were detected by ultra-violet (UV) illumination.

2.4.5 Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange® XL site-directed mutagenesis kit (Stratagene). Complementary primers were designed with the point mutations in the middle of the primers with 15-20 nucleotides of correct sequence on both sides. The following is the list of sense and antisense primers used:

1. MOAP-1 S27,29,31A

   GATTGCCGGCATCGCCCAGGCCTGCGCTGTGGCAGAAATCG (sense)

   CGATTTCTGCCACAGCGCAGGCCTGGGCGATGCCGGCAATC (antisense)
2. MOAP-1 S27,29,31D

   GATTGCCGGCATCGACCAGGACTGCGACGTGGCAGAAATCG (sense)

   CGATTTCTGCCACGTCGCAGTCCTGGTCGATGCCGGCAATC (antisense)
3. MOAP-1 S169,173A

   GCTGAGAGTGTTCGCGGGCAGGGAGGCTCCAGAACCAGG (sense)

   CCTGGTTCTGGAGCCTCCCTGCCCGCGAACACTCTCAGC (antisense)

4. MOAP-1 S169,173D

   GCTGAGAGTGTTCGACGGCAGGGAGGACCCAGAACCAGG (sense)

   CCTGGTTCTGGGTCCTCCCTGCCGTCGAACACTCTCAGC (antisense)

All point mutations were made using PXJ-HA-MOAP-1 (wild type) as the DNA template apart from MOAP-1 S27,29,31,169,173A and MOAP-1 S27,29,31,169,173D which were made using MOAP-1 S27,29,31A and MOAP-1 S27,29,31D respectively as the template. 50-100 ng of DNA template, 125 ng of each primer, 1 µl of dNTP mix [25 mM for each deoxy-adenine (dATP), deoxy-cytosine (dCTP), deoxy-guanine (dGTP) and deoxy-thymidine (dTTP) nucleotides] and 1 µl of Pfu DNA polymerase (2.5 U/µl) was used for each 50 µl reaction. The PCR reaction was performed by 16 cycles of DNA denaturation at 95 ^{o}C for 30 sec, primer annealing at 50 ^{o}C for 1 min and DNA extension at 68 ^{o}C for 2 min/1 kb after a pre-denaturation at 95 ^{o}C for 5 min. The reaction was kept at 4 ^{o}C after the last cycle. The newly synthesized and non-methylated DNA incorporated with targeted mutant was obtained by addition of 1 µl of DpnI to the reaction to digest the methylated wide type parental DNA templates. The DNA with targeted mutation was transformed into DH5α *E. Coli* using the heat shock protocol as described in Section 2.4.1. Potential DNA plasmid clones were first sequenced to identify clones with the desired mutations. The plasmid clones with the desired mutations were then sequenced from end to end to ensure that no undesired mutations were present.

2.4.6 RNA extraction, cDNA preparation and quantitative Real-time PCR

Total mRNA transcripts were extracted from HEK293T cells (transiently transfected with TRIM11 siRNAs as described in Section 2.2.5) using RNeasy® Plus Minikit (Qiagen) and treated with RNase-free DNase (Qiagen) following the supplier's protocol. The concentration of the total mRNA extracted was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Reverse transcription was performed by using Superscript First Strand synthesis system (Invitrogen), following the supplier's protocol. The resulting cDNA was used for isolating certain genes. GAPDH was used as an internal control. The cDNA was then subjected to quantitative real-time PCR (qRT-PCR) using QIAgility (Qiagen). The following primers were used in qRT-PCR:

1. TRIM11
   Forward primer: GGGAGTGTGTCCTTTGAGCA
   Reverse primer: TGCAGGAACTGTGCAGCTTA
2. GAPDH
   Forward primer: ATGTTCGTCATGGGTGTGAA
   Reverse primer: TGTGGTCATGAGTCCTTCCA

2.5 PROTEIN ALIGNMENT TOOL

The following online tool was used to align multiple protein sequences of MOAP-1 and TRIM11:

1. ClustalW2 Multiple Sequence Alignment
   http://www.ebi.ac.uk/Tools/msa/clustalw2/

CHAPTER 3

Identification and characterization of TRIM11 as a putative E3 ubiquitin ligase of MOAP-1

3.1 INTRODUCTION

3.1.1 Identification of TRIM11 and TRIM21 as close homologues of TRIM39

Previous members in the laboratory have reported the identification and characterization of TRIM39 as a binding partner of MOAP-1 (103). TRIM39 was found to extend the half-life of MOAP-1 through the inhibition of MOAP-1 poly-ubiquitination process, thereby reducing the elimination of MOAP-1 by the ubiquitin proteasome system (UPS). The increased protein level of MOAP-1 brought about by over-expression of TRIM39 sensitizes cells to the DNA-damaging agent, Etoposide (ETOP)-induced cell death, and to Bax-mediated cytochrome c release from isolated mitochondria. In a similar manner, the knockdown of TRIM39 leads to decreased stabilization of MOAP-1 as well as reduced sensitivity of the cells to ETOP-induced apoptosis. In a recent study, it was revealed that the influence of TRIM39 on MOAP-1 levels stems from the ability of TRIM39 to directly inhibit APC/C^{Cdh1}-mediated protein ubiquitination of MOAP-1 (104). However, it is not yet clear how TRIM39 inhibits the APC/C, a multi-unit E3 ubiquitin ligase.

Since TRIM family of proteins are generally believed that to function as E3 ubiquitin ligases due to the presence of the characteristic RING finger domain, it is conceivable that perhaps there could be a close homologous protein of TRIM39 that can function as an E3 ubiquitin ligase for MOAP-1. Hence, we decided to identify and evaluate close homologues of TRIM39 to assess their ability in promoting degradation of MOAP-1. To address this, we analyzed the phylogenetic tree of the TRIM family and identified TRIM11 and

TRIM21 as close homologues of TRIM39 (Fig 3.1A). Using ClustalW2, an online software for multiple protein sequence alignment, we demonstrated that human protein sequences of TRIM11 and TRIM21 share up to 40% amino acid identity to TRIM39 (Fig 3.1B).

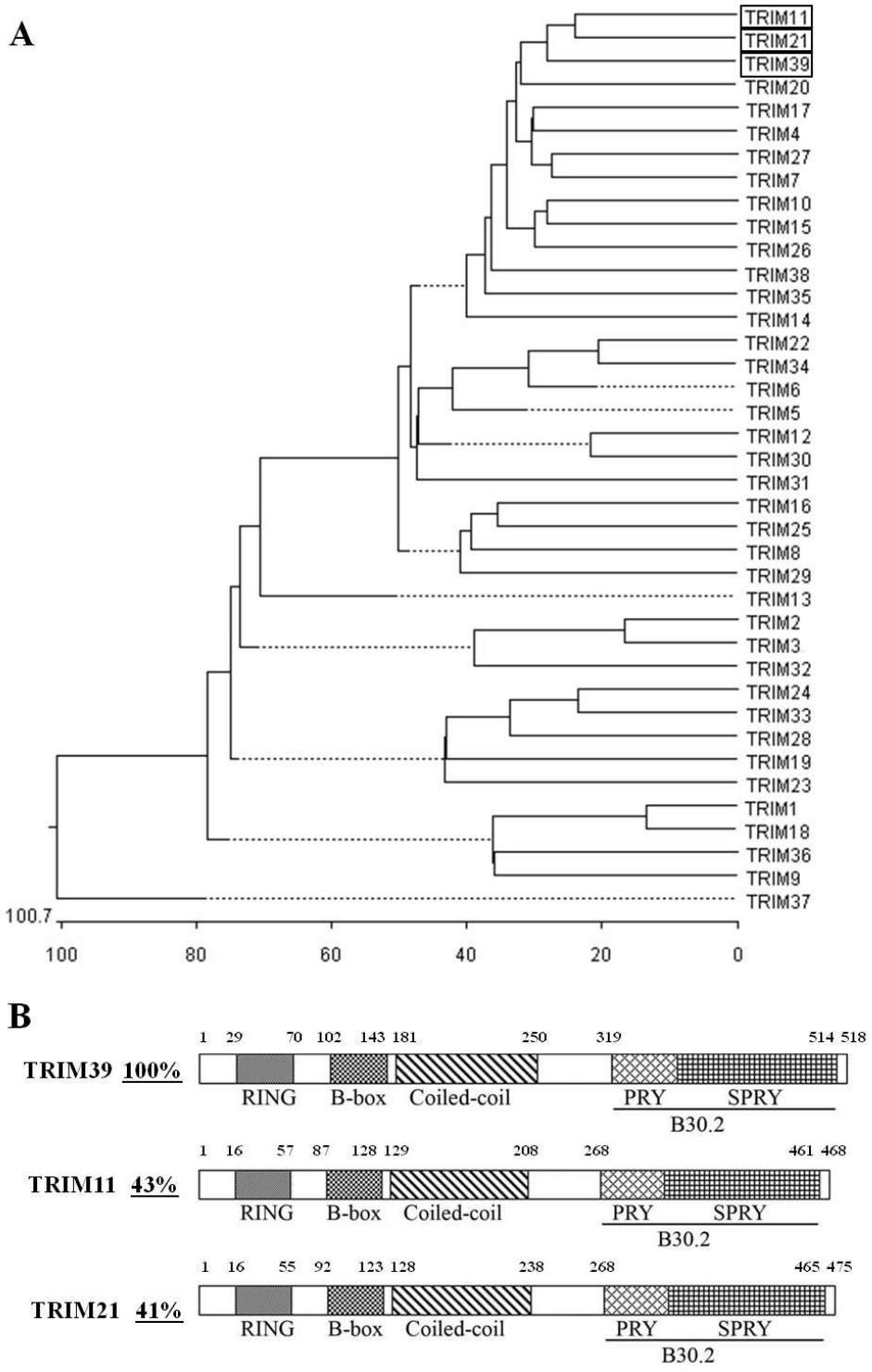

Figure 3.1 TRIM family of proteins. A. Phylogenetic tree analysis of the TRIM family of proteins. TRIM11 and TRIM21 are close homologues of TRIM39. **B.** Schematic diagram to depict full length human TRIM39, TRIM11 and TRIM21 proteins. The TRIM family of proteins is characterized by a tripartite (also known as RBCC) motif that comprises a RING finger domain, a B-box and a coiled-coil domain at the N-terminal region (187) The C-terminus consists of the B30.2 domain which comprises of PRY and SPRY domains. The percentage (underlined) reflects the degree of homology between TRIM39, TRIM11 and TRIM21 based on their protein sequences. the amino acid identity to TRIM39. The amino acid residues which demarcate the various domains are indicated.

3.2 RESULTS

3.2.1 TRIM11, but not TRIM21, down-regulates protein levels of MOAP-1

Since we postulate that there could be a close homologous protein of TRIM39 that may potentially function as an E3 ubiquitin ligase of MOAP-1, we first evaluated whether TRIM11 could negatively modulate the protein stability of MOAP-1 through the ubiquitin proteasome degradation pathway. To this end, we examined whether co-expression of TRIM11 and MOAP-1 will down-regulate protein level of MOAP-1 and, if so, whether the proteasome inhibitor MG132 will inhibit the TRIM11-induced MOAP-1 down-regulation. Co-expression of TRIM39 and MOAP-1 was included in this experiment as a control to validate the up-regulation of MOAP-1 by TRIM39. HEK293T cells were used due to its high transfection efficiency.

In agreement with the results obtained previously (103), TRIM39 up-regulated protein level of MOAP-1 under co-expression condition (Fig 3.2, lanes 1 and 4). On contrary, TRIM11 down-regulated MOAP-1 and proteasome inhibitor (lanes 2 and 4), MG132 reversed the TRIM11-mediated down-regulation of MOAP-1 (lanes 2 and 3). These results suggest TRIM11 mediates the protein stability of MOAP-1 in a manner that is dependent on the proteasome. In addition, both MOAP-1 and TRIM11 are up-regulated by MG132, indicating that the protein stability of both proteins is regulated via the ubiquitin proteasome system (UPS).

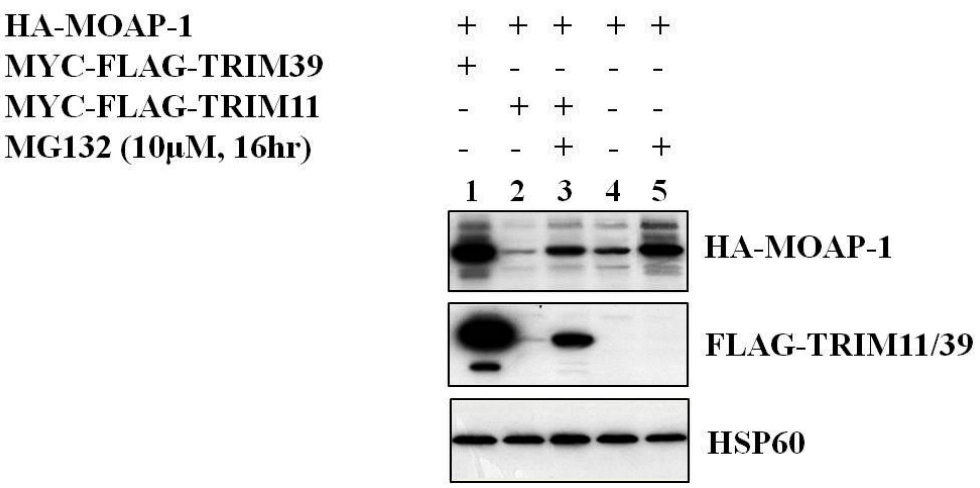

Figure 3.2 TRIM11 and TRIM39 exert opposing effect on the protein stability of MOAP-1. HEK293T cells are transiently transfected with HA-MOAP-1 and MYC-FLAG-TRIM11 or MYC-FLAG-TRIM39. At 24 hour post-transfection, 10μM MG132 was added to the cells for 16 hours. Cells were harvested and protein samples were separated on SDS-PAGE and analyzed by western blotting. 50μg total cell lysate were loaded in each lane. HSP60 was used as a loading control.

We next proceeded to check whether TRIM21 could negatively modulate the protein stability of MOAP-1 through the ubiquitin proteasome degradation pathway. To this end, we examined whether co-expression of TRIM21 and MOAP-1 will down-regulate MOAP-1 and, if so, whether the proteasome inhibitor MG132 will inhibit the TRIM21-induced MOAP-1 down-regulation. Co-expression of TRIM11 and MOAP-1 was included in this experiment to validate the down-regulation of MOAP-1 by TRIM11.

Consistent with the result obtained in Fig 3.2, TRIM11 down-regulated MOAP-1 (Fig 3.3, lanes 3 and 5) and MG132 reversed the TRIM11-mediated down-regulation of MOAP-1 (lanes 3 and 4). In contrast, over-expression of TRIM21 did not result in the down-regulation of MOAP-1 (lanes 5 and 9). To a certain extent, an up-regulation of MOAP-1 by TRIM21 could be observed suggesting that TRIM21 may function, similar to TRIM39, as a positive regulator of MOAP-1. In addition, the protein level of TRIM21 was not

affected by MG132 treatment, suggesting that the protein regulation of TRIM21 is likely not mediated through the UPS. However, since the objective of the study was to identify a close homologue of TRIM39 that could potentially function as an E3 ubiquitin ligase of MOAP-1, we decided to focus the study on the regulation of MOAP-1 by TRIM11 instead of TRIM21.

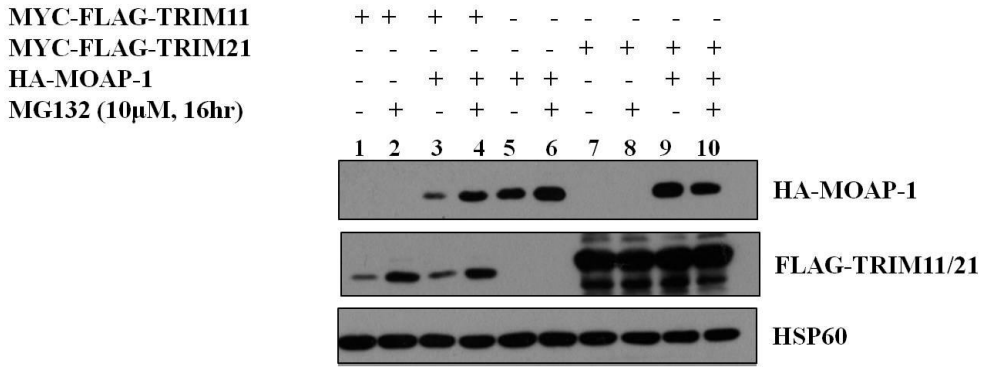

Figure 3.3 TRIM11, but not TRIM21, down-regulates MOAP-1 protein level under co-expression condition in HEK293T cells. HA-MOAP-1 was co-transfected with MYC-FLAG-TRIM11 or MYC-FLAG-TRIM21 in HEK293T cells. At 24 hour post-transfection, 10μM MG132 was added to the cells for 16 hours. Cells were harvested and protein samples were separated by SDS-PAGE and analyzed with western blotting. 50μg total cell lysate were loaded in each lane. HSP60 was used as a loading control.

3.2.2 TRIM11 down-regulates PNMA2, PNMA4 (MOAP-1) and PNMA6

MOAP-1, also known as PNMA4, belongs to the Paraneoplastic Ma antigens (PNMA) family which comprises of six members, PNMA1-6 in human. Notably, PNMA1-3 were first identified through screening expression libraries using sera from patients suffered from Paraneoplastic Neurological Syndromes (PNS). Whereas, MOAP-1 along with PNMA5 and PNMA6, were found to share high degree of amino acid sequence homology with PNMA1-3

(40-54% amino acid identity), indicating that they are members of the same protein family (Fig 3.4) (188).

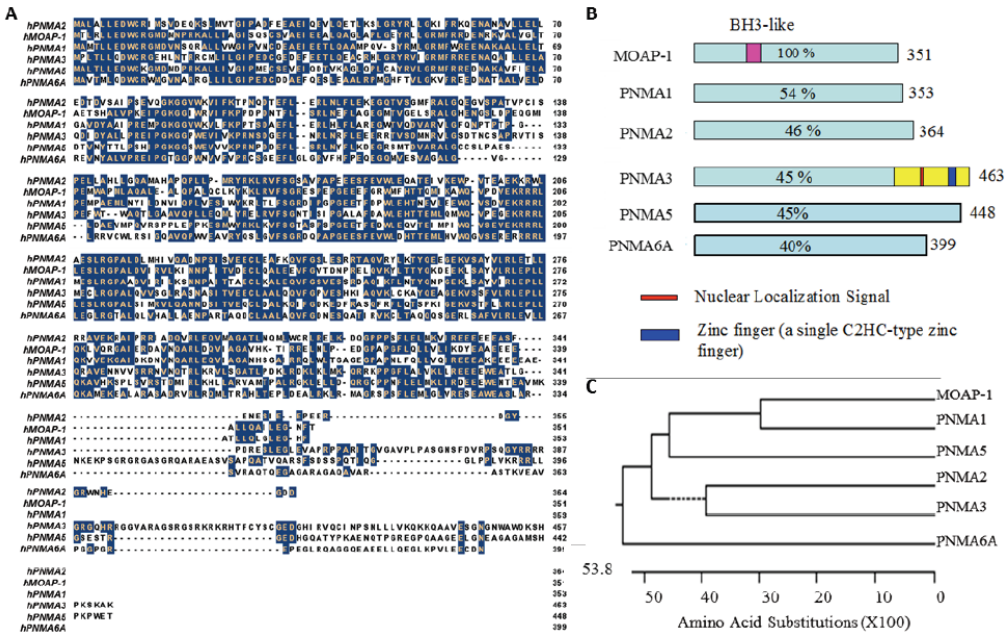

Figure 3.4 MOAP-1 (PNMA) family of proteins. A. Alignment of predicted amino acid sequences of MOAP-1 (PNMA) family of proteins. **B.** Schematic to depict MOAP-1 family of proteins. Percentage represents amino acid identity to MOAP-1. Total number of amino acid for the full length protein is indicated on the right. **C.** Phylogenetic tree analysis of the MOAP-1 family of proteins.

We asked if down-regulation of MOAP-1 (PNMA4) by TRIM11 is specific to MOAP-1. To do this, PNMA1-6 and TRIM11 were transiently co-transfected into HEK293T cells for 48 hours. As shown in Fig, 3.5, apart from MOAP-1 (PNMA4), TRIM11 also down-regulates the protein levels of PNMA2 and PNMA6. Interestingly, other members in the laboratory have demonstrated that PNMA2 and PNMA6 were the only PNMA members found to interact with MOAP-1 (data not shown). It may suggest that PNMA2, 4 and

6 could exist as a complex and hence when targeted by TRIM11, all three members are concurrently down-regulated.

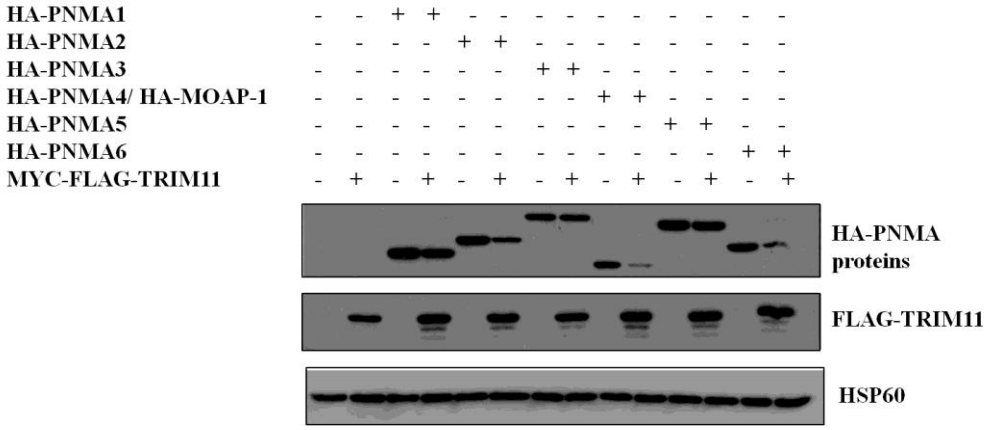

Figure 3.5 TRIM11 down-regulates PNMA 2, 4 and 6 under co-expression condition in HEK293T cells. HEK293T cells were transiently transfected with HA-PNMA1-6 and MYC-FLAG-TRIM11 for 48 hours. Cells were harvested and protein samples were separated by SDS-PAGE and analyzed with western blotting. 50μg total cell lysate were loaded in each lane. HSP60 was used as a loading control.

3.2.3 TRIM11 interacts with MOAP-1

Since TRIM11 is able to down-regulate protein levels of MOAP-1, we next proceeded to ask if a physical association between them can be detected. To do so, an *in vitro* pull-down assay was conducted. Bacterially expressed FLAG-MOAP-1 was incubated with bacterially expressed GST or GST-TRIM11. As illustrated in Fig 3.6, direct interaction between MOAP-1 and TRIM11 was detected. This supports the hypothesis that TRIM11 may function as an E3 ubiquitin ligase of MOAP-1 since direct association between the E3 ligase and its substrate was shown.

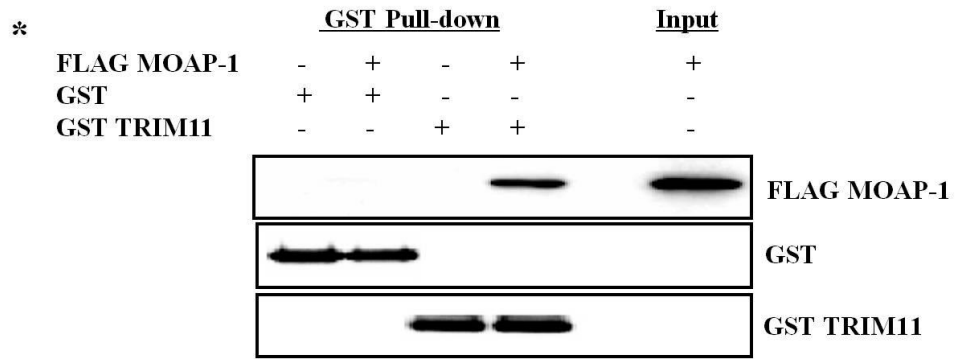

Figure 3.6 TRIM11 interacts with MOAP-1 *in vitro*. Bacterially produced FLAG-MOAP-1 was incubated with bacterially expressed GST or GST-TRIM11. Associated MOAP-1 and input MOAP-1 were determined by immuno-blotting with anti-FLAG antibody. GST and GST-TRIM11 was detected with anti-GST antibody. The asterisk indicates that the data was provided by a collaborator from Xiamen University.

In addition, a co-immuno-precipitation assay was carried out in HEK29T cells over-expressing MYC-FLAG-TRIM11 and HA-MOAP-1. At 24 hour post-transfection, 10μM MG132 was added to the cells for 16 hours. Cell lysates were immuno-precipitated with anti-FLAG antibody. As illustrated in Fig 3.7, MOAP-1 was found to interact with TRIM11 under MG132 treatment *in vivo*, further supporting the hypothesis that TRIM11 may function as an E3 ubiquitin ligase of MOAP-1..

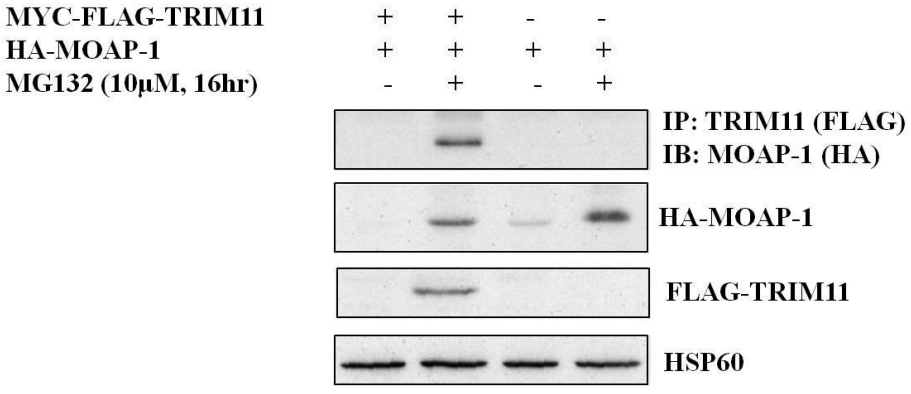

Figure 3.7 TRIM11 interacts with MOAP-1. HEK293T cells were transiently transfected with MYC-FLAG-TRIM11 and HA-MOAP-1 for 24 hours. 10μM MG132 was added to the cells for 16 hours. Cells were then harvested and cell lysates were immuno-precipitated with anti-FLAG. Cell lysates and immuno-precipitates were separated by SDS-PAGE and analyzed with western blotting. HSP60 was used as a loading control.

3.2.4 TRIM11 regulates MOAP-1 by modulating its protein stability

Using the cycloheximide (CHX) chase assay, it was revealed that TRIM39 up-regulates MOAP-1 protein level by regulating its protein stability (103). We postulated that TRIM11 may regulate MOAP-1 abundance in a similar manner. To test this possibility, we evaluated the effect of TRIM11 on the half-life of MOAP-1. 100μg/ml CHX was added to HEK293T cells transiently over-expressing both HA-MOAP-1 and MYC-FLAG-TRIM11. Steady-state protein levels of HA-MOAP-1 were assessed at 1, 3 and 5 hours after treatment of CHX. As shown in Fig 3.8, MOAP-1 was rapidly degraded in cells over-expressing TRIM11 than in vector-transfected cells suggesting that TRIM11 down-regulates MOAP-1 level by regulating its protein stability.

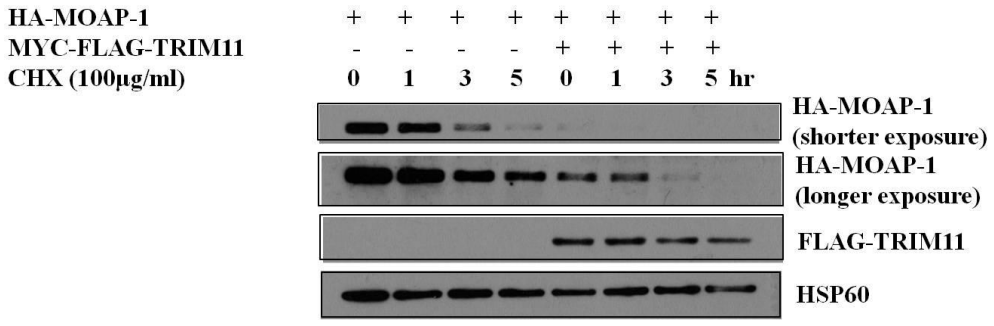

Figure 3.8 TRIM11 destabilizes MOAP-1. Cycloheximide (CHX) chase analysis of MOAP-1 turn-over. HEK293T cells were transfected with HA-MOAP-1 and MYC-FLAG-TRIM11. Cells were subjected to 100μg/ml cycloheximide at 24 hour post-transfection and harvested at the indicated time points. HSP60 was used as a loading control to demonstrate that equal amounts of cell lysates was added into each lane.

3.2.5 RING finger domain of TRIM11 is required for TRIM11-mediated down-regulation of MOAP-1

Given that TRIM11 has a RING finger domain and is a potential E3 ubiquitin ligase of MOAP-1, we investigated the importance of the RING

finger domain in the down-regulation of MOAP-1 by TRIM11 since critical residues in the RING finger domain have been shown to be essential for mediating E3 ubiquitin ligase activity (189). The RING finger domain which comprises of a series of cysteine and histidine residues forms a 3-dimensional cross-braced structure held together by zinc ions, mediating protein-protein interactions. Substitution of cysteine and histidine residues to alanine disrupts the conformation of the RING finger domain leading to the loss of enzymatic activity and protein interaction with other proteins (104). Multiple site-directed mutagenesis was carried out to generate two versions of human TRIM11 in which conserved cysteine and histidine residues at 16, 19, 31 and 33 were substituted with alanine (Fig 3.10). It is noteworthy that all the four residues selected are conserved in all mammalian species examined (Fig 3.9, boxed in full lines). The three other conserved cysteine residues (at 36, 39 and 43) found in the RING finger domain were not included in this study (Fig 3.9, boxed in dotted lines).

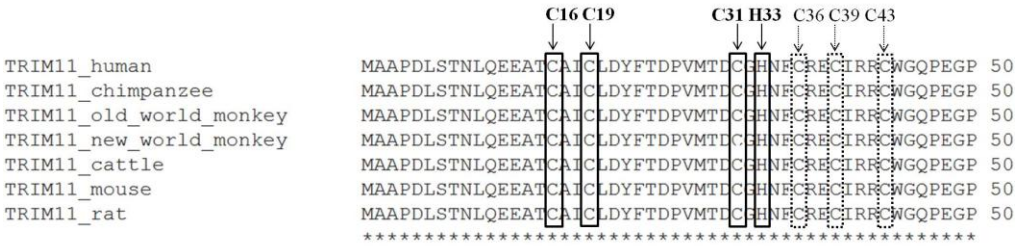

Figure 3.9 Identification of conserved residues for multi-site directed mutagenesis. Multiple protein sequence alignment was carried using ClustalW2 algorithm. Cysteine (C) residues at 16, 19 and 31 and histidine (H) residue at 33 were substituted with alanine to investigate the importance of the RING finger domain in the down-regulation of MOAP-1 by TRIM11 (boxed in full lines). Conserved cysteine residues at 36, 39 and 43 in the RING finger domain remain to be mutated in future work (boxed in dotted lines).

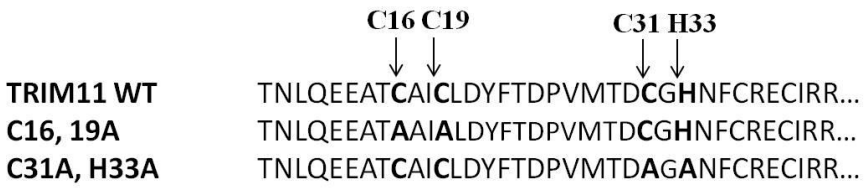

Figure 3.10 TRIM11 mutants with point mutations in the RING finger domain. Each mutant is listed and differences from the WT sequence are indicated in bold.

Mutations of the cysteine and histidine residues within the RING finger domain of TRIM11 seem to reduce drastically the ability of TRIM11 to down-regulate MOAP-1 (Fig 3.11), suggesting that these key residues in the RING finger domain are required for TRIM11-mediated down-regulation of MOAP-1. In addition, we observed that these TRIM11 cysteine mutants, unlike the wild type TRIM11, were more stable and MG132 had little, if any, effect on the stability of these TRIM11 mutants (Fig 3.11). This may suggest that the RING finger domain of TRIM11 may be required for its auto-regulation by the ubiquitin proteasome system. Similar finding was reported for TRIM5, another member of the TRIM/RBCC family. TRIM5 mutants containing amino acid changes designed to disrupt the zinc binding of the RING finger mutant were observed to be more stable suggesting that the presence of an intact RING finger domain contributes to the degradation of TRIM5 (190).

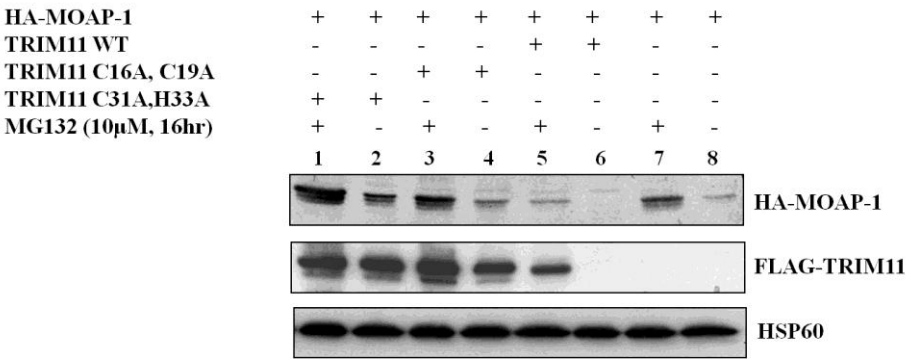

Figure 3.11 Mutation of the conserved cysteines in the RING domain of TRIM11 loses its ability to degrade MOAP-1. HEK293T cells were transiently transfected with MYC-FLAG-TRIM11 WT and mutants (C16,19A and C31A,H33A). At 24 hour post-transfection, 10μM MG132 was added to the cells for 16 hours. Cells were then harvested and protein samples were separated by SDS-PAGE and analyzed with western blotting. 50μg total cell lysate were loaded in each lane. HSP60 was used as a loading control.

3.2.6 TRIM11 interacts with E2 ubiquitin-conjugating enzymes

In some instances, it is known that the RING-type E3 ubiquitin ligase would need to interact with E2 ubiquitin-conjugating enzymes for mediating the transfer of ubiquitin from the active site of the E2 enzymes to (usually) lysine residues on substrate proteins (94). Thus, we asked if TRIM11, being a classical RING-type E3 ubiquitin ligase can interact with E2 ubiquitin-conjugating enzymes UbcH2 and UbcH13. It was reported that UbcH2 accepts ubiquitin from E1 ubiquitin-activating enzyme and catalyzes lysine-11- and lysine-48-linked poly-ubiquitination (191). UbcH13, in contrast, catalyzes the synthesis of the non-canonical lysine-63-linked poly-ubiquitin chains which typically does not result in the degradation of the protein by the UPS (192,193). HEK293T cells were transiently transfected with TRIM11 and UbcH2 or UbcH13. At 24 hour post-transfection, 10μM MG132 was added to the cells for 16 hours. Cells were harvested and cell lysates were subjected to

immuno-precipitation with anti-FLAG antibody. As illustrated in Fig 3.12, TRIM11 was found to interact with both E2 ubiquitin-conjugating enzymes that were capable of catalyzing the two main forms of poly-ubiquitination (namely lysine-48- and lysine-63-linkages). This may further support the notion that TRIM11 can interact with E2 ubiquitin-conjugating enzymes to potentially module the proteasome-mediated degradation of MOAP-1.

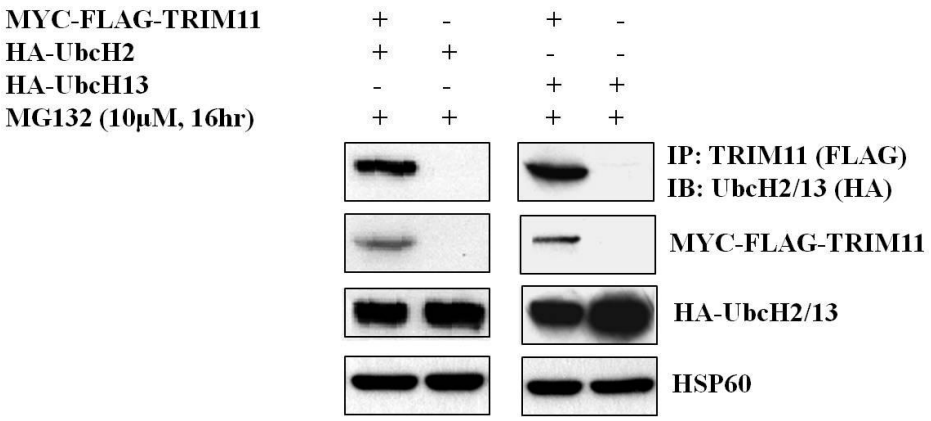

Figure 3.12 TRIM11 interacts with E2 ubiquitin-conjugating enzymes, UbcH2 and UbcH13. HEK293T cells were transiently transfected with MYC-FLAG-TRIM11 and HA-UbcH2 or HA-UbcH13 for 24 hours. 10 μM MG132 was then added to the cells for 16 hours. Cells were then harvested and cell lysates were immuno-precipitated with anti-FLAG. Cell lysates and immuno-precipitates were separated by SDS-PAGE and analyzed with western blotting. HSP60 was used as a loading control.

3.2.7 TRIM11 catalyzes the poly-ubiquitination of MOAP-1 *in vitro*

Since TRIM11 was shown to interact with and degrade MOAP-1, we next asked if direct E3 ubiquitin ligase activity of TRIM11 on MOAP-1 can be detected. To do so, we performed *in vitro* ubiquitination assay. In addition, GST-TRIM11 RING finger domain mutants (C16,19A and C31A,H33A) were used to validate if the RING finger domain of TRIM11 is required for the poly-ubiquitination of MOAP-1. In brief, FLAG-MOAP-1 and GST-TRIM11

[wild type (WT), C16,19A or C31A,H33A mutants] purified from bacteria were incubated with recombinant E1 enzyme, recombinant E2 ubiquitin-conjugating enzyme (UbcH5C) and ubiquitin. UbcH5C was used as it is commonly used for *in vitro* ubiquitination reactions and is one the most active class of E2 ubiquitin-conjugating enzymes in cell extracts (194). Similar to UbcH2, UbcH5C was reported to accept ubiquitin from the E1 ubiquitin-activating enzyme and catalyzes lysine-11- and lysine-48-linked poly-ubiquitination (195). After the incubation, FLAG-MOAP-1 was solubilized and analyzed by western blotting using anti-ubiquitin antibody to detect ubiquitinated FLAG-MOAP-1 (Fig 3.13A). In a similar manner, for Fig 3.13B, reaction samples were boiled in 1% SDS to stop the reaction and then diluted ten times in 0.5% TNTE. The reaction samples were precipitated with anti-FLAG antibody and analyzed by western blotting using anti-ubiquitin antibody to detect ubiquitin-conjugated FLAG-MOAP-1.

As illustrated in Fig 3.13, a clear accumulation of poly-ubiquitinated MOAP-1 products was observed in the reaction mixture containing GST-TRIM11, FLAG-MOAP-1, E1 enzyme, UbcH5C and ubiquitin (lane 4; Fig 3.13A for total and Fig 3.13B for IP). While, in reaction mixtures where GST-TRIM11 WT was substituted with the catalytically inactive mutants (C16,19A and C31A,H33A), the poly-ubiquitination of MOAP1 was abolished (lanes 5 and 6 respectively). These results suggest that the *in vitro* poly-ubiquitination of FLAG-MOAP-1 is catalyzed by TRIM11 and E2 ubiquitin-conjugating enzyme, UbcH5C. In addition, the RING finger domain of TRIM11 is essential and required for the poly-ubiquitination of MOAP-1 by TRIM11.

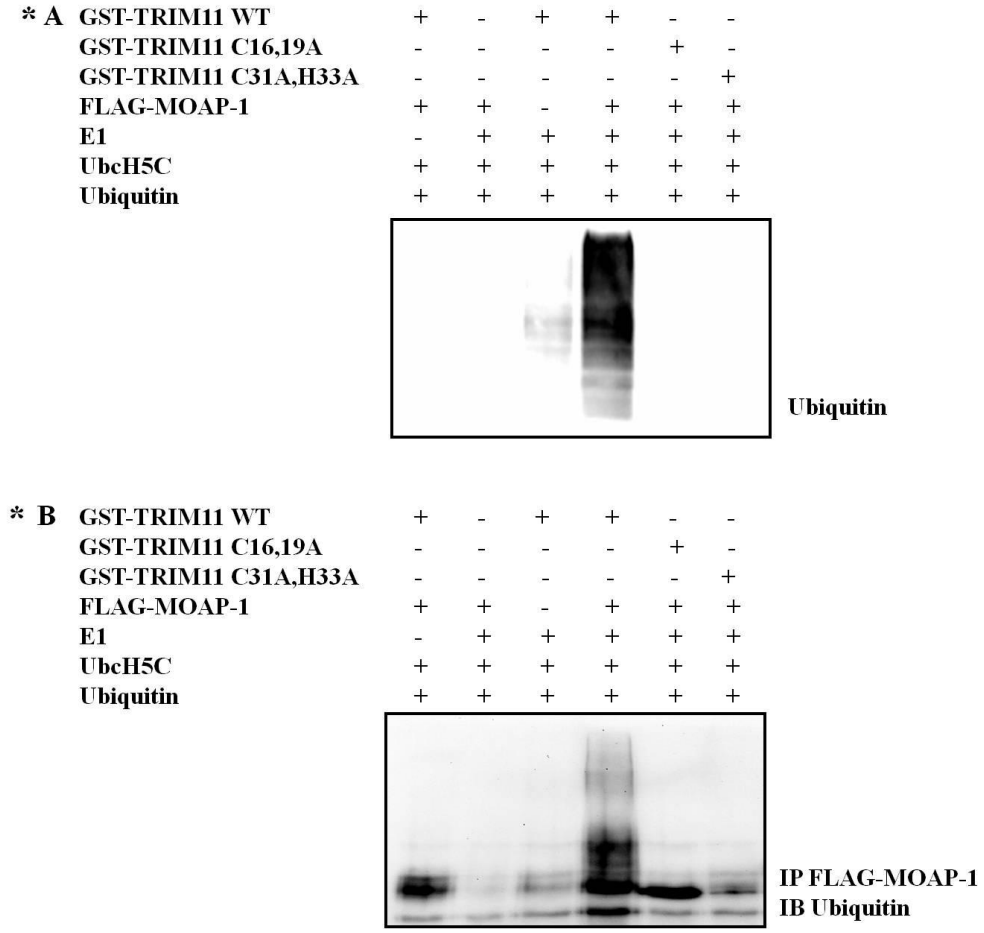

Figure 3.13 TRIM11 and UbcH5C catalyzed the *in vitro* poly-ubiquitination of MOAP-1 and the RING finger domain of TRIM11 is required for this poly-ubiquitination of MOAP-1 by TRIM11 (A) FLAG-MOAP1 and GST-TRIM11 [wild type (WT), C16,19A or C31A,H33A mutants] purified from bacteria were subjected to an *in vitro* ubiquitination assay. Samples were boiled in loading buffer to stop the reaction and loaded to SDS-PAGE directly for a western blotting assay. **(B)** Reaction samples were boiled in 1% SDS to stop the reaction, then diluted 10 times in 0.5% TNTE and precipitated with anti-FLAG antibody. Ubiquitin-conjugated MOAP-1 was then detected with anti-Ub antibody**.** The asterisk indicates that the data was provided by a collaborator from Xiamen University.

3.2.8 Knock-down of endogenous TRIM11 in HEK293T does not alter the abundance of MOAP-1

We have now shown that TRIM11 over-expression shortened the protein half-life of MOAP-1and TRIM11 possesses E3 ubiquitin ligase activity to ubiquitylate MOAP-1 *in vitro*. We next determined whether siRNA

knock-down of TRIM11 can increase the steady-state levels of endogenous MOAP-1 in HEK293T cells. Since we do not yet have a good antibody that can detect endogenous TRIM11 protein levels, real-time PCR was conducted to evaluate the knock-down efficiency of TRIM11. To do this, we transfected 10 nM scrambled control siRNA and TRIM11 siRNA1-4 into HEK293T cells for 72 hours. Cells were then harvested for RNA isolation followed by reverse transcription to obtain the cDNA. The cDNA was then subjected to real-time PCR to quantify the mRNA levels of TRIM11. All four siRNAs of TRIM11 reduced at least 50% of TRIM11 mRNA levels (Fig 3.14A).

We then evaluated whether knock-down of endogenous TRIM11 can increase the steady-state levels of MOAP-1 in HEK293T cells. 10nM scrambled control siRNA and TRIM11 siRNA1-4 were transfected into HEK293T cells for 72 hours. As shown in Fig 3.14B, knockdown of endogenous TRIM11 did not increase the steady-state abundance of endogenous MOAP-1 in HEK293T cells. This may be due to low endogenous expression of TRIM11 in HEK293T cells resulting in negligible difference in protein abundance of MOAP-1 between the TRIM11 knock-down cells and control vector cells. In addition, similar results were obtained at 20 nM of TRIM11 siRNAs (data not shown). Due to concerns over off-target effects in which siRNAs may influence the expression of non-homologous or partially homologous gene targets (196,197), the knock-down analysis was not conducted at higher siRNAs concentrations.

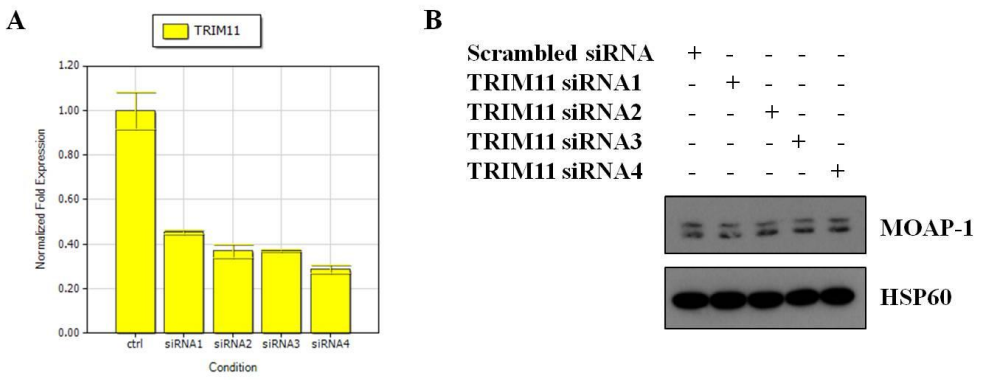

Figure 3.14 Knockdown of endogenous TRIM11 by siRNA did not alter MOAP-1 abundance in HEK293T cells. (A) Scrambled control siRNA and TRIM11 siRNA1-4 were transfected into HEK293T cells for 72 hours. Cells were then harvested for RNA isolation followed by reverse transcription to obtain the cDNA. The cDNA was then subjected to real-time PCR to quantify the mRNA expression of TRIM11. (B) Scrambled control siRNA and TRIM11 siRNA1-4 were transfected into HEK293T cells for 72 hours. Cells were then harvested and subjected to SDS-PAGE and analyzed by western blotting. Data shown are for immuno-blot analyses of MOAP-1 and loading control, HSP60.

3.3 Discussion

Characterized by a tripartite (also known as RBCC) motif that comprises a RING finger domain, a B-box and a coiled-coil domain at the N-terminal region, the TRIM family of proteins have been shown to be involved in a number of critical intra-cellular processes including apoptosis, differentiation, cell differentiation and tumorigenesis (187). Several TRIM family members have been reported to demonstrate E3 ubiquitin ligase activity through their RING domain. In addition, some TRIM proteins have been reported to be involved in the regulation of the immune system but their exact mechanism of action remains to be elucidated (198,199). TRIM11 contains the RBCC domain and a C-terminal B30.2/SPRY domain (Fig. 3.1B). Recent studies have demonstrated the role of TRIM11 in innate immunity where it

was shown to be a negative regulator of type I interferons (IFNα/β) production (200). In addition, TRIM11 has also been demonstrated to act in a RING domain-dependent manner to reduce the levels of TRIM5α protein, an inhibitor of HIV infection (201). As for its role as a E3 ubiquitin ligase, the following substrates have been identified: Humanin, a 24-amino-acid neuro-protective peptide; activator-recruited co-factor 105-kDa component (ARC105), a component of the ARC complex that mediates chromatin-directed transcription factors; and Pax6, a member of the paired-box homeo-domain transcription factors; and PHOX2B, a paired-box homeo-domain transcription factor (202-205).

Since it has been reported that higher levels of MOAP-1 in cancers cells are sufficient to enhance their sensitivity to chemotherapeutic drugs, targeting the E3 ubiquitin ligases of MOAP-1 to inhibit ubiquitin-mediated degradation and up-regulate MOAP-1 protein levels holds considerable therapeutic potential. Recent studies have also demonstrated that TRIM11 functions as a RING E3 ubiquitin ligase degrading Humanin, ARC105, Pax6 and PHOX2B (202-205). In this study, we demonstrated an important role for TRIM11, a member of the TRIM family of RING finger proteins, in regulating the ubiquitination and proteasome-mediated degradation of MOAP-1 protein.

Unlike TRIM39, a close homologue of TRIM11, which has been reported to stabilize MOAP-1, TRIM11 interacts with and degrades MOAP-1 through the ubiquitin proteasome system (Figs 3.2, 3.3, 3.6 and 3.7). Furthermore, mutation of conserved cysteine and histidine residues at 16, 19, 31 and 33 in the RING finger domain of TRIM11 abrogated its ability to degrade MOAP-1 (Fig 3.11), suggesting that the RING finger domain of

TRIM11 is required for regulating the protein stability of MOAP-1. In addition, using *in vitro* ubiquitination assay, we showed direct poly-ubiquitinating activity of TRIM11 on MOAP-1 (Fig 3.13).

However, knock-down of endogenous TRIM11 in HEK293T cells by siRNAs did not increase the steady-state abundance of endogenous MOAP-1 (Fig 3.14). This may be due to low endogenous expression of TRIM11 in HEK293T cells resulting in negligible difference in MOAP-1 abundance between the TRIM11-knockdown and vector control cells. Hence, more cell-lines will need to be screened in the future to identify the cell-lines with higher endogenous levels of TRIM11.

Collectively, this study suggests that TRIM11 may function as a putative E3 ubiquitin ligase of MOAP-1. To date, the APC/C^{Cdh1} has also been suggested to function as a MOAP-1 E3 ubiquitin ligase (104). However, it is likely that both proteins function as E3 ubiquitin ligases of MOAP-1 under different context (i.e. cell type, cell condition). It is also possible that TRIM11 and APC/C^{Cdh1} act on different post-translational modified forms of MOAP-1.

CHAPTER 4

Evaluation of phosphorylation as a regulatory mechanism to modulate protein stability of MOAP-1

4.1 INTRODUCTION

Characterization study conducted by our laboratory has revealed that MOAP-1 is a short lived protein with a half-life of approximately 25 mins and it is constitutively degraded by the ubiquitin proteasome system (UPS) under non-apoptotic condition (67). On contrary, MOAP-1 is rapidly up-regulated by multiple apoptotic stimuli including endoplasmic reticulum (ER) stress inducer Thapsigargin (THA), DNA-damaging agents Etoposide (ETOP), serum withdrawal and death receptor TNF-related apoptosis-inducing ligand (TRAIL) (67). Similarly, the responsiveness of MOAP-1 up-regulation induced by different apoptotic stimuli differs in different cell-lines. For instance, the up-regulation of MOAP-1 by TRAIL and THA is most robust in HCT116, a colorectal cancer cell-line while the elevation of MOAP-1 by ETOP is best observed in HEK293T cell-line (67). Notably, pre-treatment of ETOP followed by proteasome inhibition by MG132 reduced the accumulation of ubiquitinated forms of MOAP-1 suggesting that the effect of apoptotic stimuli on stabilizing MOAP-1 is likely to be mediated through the inhibition of its poly-ubiquitination process (67). Although it was shown that post-translational instead of transcriptional or translational mechanisms plays a significant role in mediating the stabilization of MOAP-1 under apoptotic condition (67), the link between apoptotic stimuli and inhibition of MOAP-1 poly-ubiquitination is still unknown.

Phosphorylation is a widely used regulatory mechanism in biological systems to control cellular homeostasis. As a transient and reversible post-translational modification, phosphorylation functions as a form of spatial and temporal regulation on macromolecules including the Bcl-2 family of proteins

such as Bim. Bim is one of the BH3-only pro-apoptotic proteins that activates Bax/Bak directly and inhibits anti-apoptotic proteins such as Bcl-2, Bcl-xL and Mcl-1, thereby releasing Bax/Bak to trigger the mitochondrial-mediated apoptotic pathway. Bim was found to undergo both post-translational modifications of ubiquitination and phosphorylation. Interestingly, phosphorylation of Bim at serine 65 by ERK was required for its association and degradation by its E3 ubiquitin ligase, TRIM2 (102). Since MOAP-1 has been shown to be regulated by the ubiquitin proteasome system, we hypothesized if MOAP-1 undergoes a similar regulation to Bim whereby phosphorylation of it may be required for its degradation via the UPS. While phosphorylation may modulate many aspects of a protein such as its intrinsic functional activity, folding and conformation, oligomerization state, sub-cellular localization, protein-protein interaction, the scope of my thesis will look specifically at the potential role of phosphorylation in regulating the protein stability of MOAP-1.

4.2 RESULTS

4.2.1 *In vitro* phosphorylation assay of GST-MOAP-1

In an attempt to determine the phosphorylation status of MOAP-1, a collaboration was set up with Jeffery Yong (Institute of Molecular and Cell Biology, Biopolis, Singapore) to conduct *in vitro* kinase assay using [γ-^{32}P] ATP. Recombinant GST-MOAP-1 was expressed in bacterial strain BL21 (DE3) and purified using glutathione sepharaose beads. The brain and liver from mouse were chosen as crude sources of protein kinase(s) to phosphorylate GST-MOAP-1 in the *in vitro* kinase assay. The brain has always been an organ of interest for the study of MOAP-1. MOAP-1 protein is found to be highly enriched in the brain whereas in other tissues, MOAP-1 exists in very low abundance, presumably due to its constitutive regulation by the UPS. In addition, unpublished data from our laboratory suggest that MOAP-1 play an important role in Jo-2 mediated apoptosis in the liver. Hence, we were keen to evaluate if these organs possess potential kinases that are capable of phosphorylating recombinant GST-MOAP-1 in the *in vitro* kinase reaction. Brain and liver were harvested and purified using ion exchange chromatography (IEX). Briefly, IEX separates proteins based on their net surface charge through electrostatic interactions that occur between proteins and the charged column. "Salting out", using increasing concentrations of salt solution (NaCl used in our case) is then used to release the proteins bound to the column. The column having higher attraction for the charged salts rather than the charged proteins, will release the protein in favour of binding to the salts instead. Correspondingly, proteins with weaker ionic interactions will elute first at lower salt concentration while higher salt concentrations will be

required to release proteins with stronger ionic interactions to the column. The purified elutes were then collected at 0.25M, 0.5M, 0.75M and 1M of NaCl. For the *in vitro* kinase reaction, GST-MOAP-1 was incubated with 10μM [γ-^{32}P] ATP and the respective purified elutes for 30min at 30^{o}C. Myelin basic protein (MBP) is a substrate for phosphorylation by several different protein kinases and was used as a positive control for the *in vitro* kinase assay.

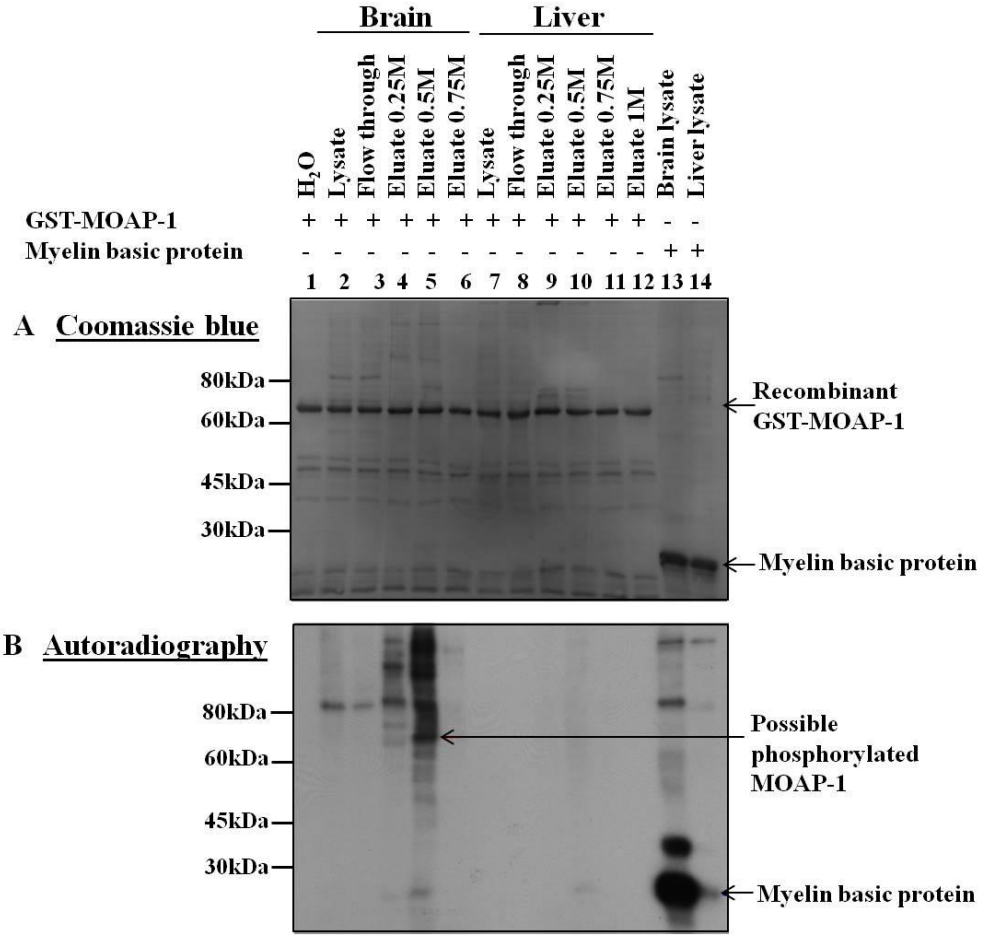

Figure 4.1 Autoradiography showed a band corresponding to the molecular weight of GST-MOAP-1 in the 0.5M brain elute reaction. A. Coomassie staining of the gel after SDS-PAGE electrophoresis. Bands corresponding to GST-MOAP-1 and myelin basic protein (MBP) were observed (indicated with arrows). **B.** Autoradiography of the membrane showed a potential band corresponding to the molecular weight of GST-MOAP-1 (lane 5). For *in vitro* phosphorylation reaction, recombinant GST-MOAP-1 was incubated with 10 μM [γ-^{32}P] ATP and the respective purified elutes for 30 min at 30 ^{o}C. The reactions were stopped by boiling in 2x SDS-PAGE sample buffer. Samples were separated on SDS-PAGE, stained with Coomassie blue and transferred to polyvinylidene difluoride membrane (PVDF) for autoradiography.

Based on the autoradiography obtained, the *in vitro* kinase reaction from 0.5M brain elute showed a band corresponding to the molecular weight of GST-MOAP-1 (Fig 4.1B, lane 5), suggesting that recombinant GST-MOAP-1 may be phosphorylated by unknown protein kinase(s) present in the 0.5M brain elute. While the band corresponding to molecular weight of myelin basic protein (MBP) gave a strong signal in the presence of brain lysate, suggesting that the *in vitro* kinase reaction worked as expected and MBP was phosphorylated by unknown protein kinase(s) found in the brain lysate during the *in vitro* kinase reaction (lane 13). However, it was observed that no band corresponding to the molecular weight of MBP was detected in the liver sample (lane 14). This could imply that there is low abundance of active protein kinases present in the liver lysate or the conditions for the harvest and purification of the liver organ were not yet optimized, leading to substantial protein degradation and the subsequent loss of enzymatic activity of the protein kinases.

In an attempt to increase the signal for the putative phosphorylation signal of GST-MOAP-1 in the 0.5M brain lysate, we repeated the ion exchange column purification process for the 0.5M brain lysate elute. Similar to the first purification process, the elutions were collected at 0.25M, 0.5M and 0.75M of NaCl. The *in vitro* kinase assay was repeated with recombinant GST-MOAP-1, doubled purified brain elutes and 10μM [γ-^{32}P] ATP for 30min at 30^{o}C. MBP was used as the positive control for the *in vitro* kinase reaction.

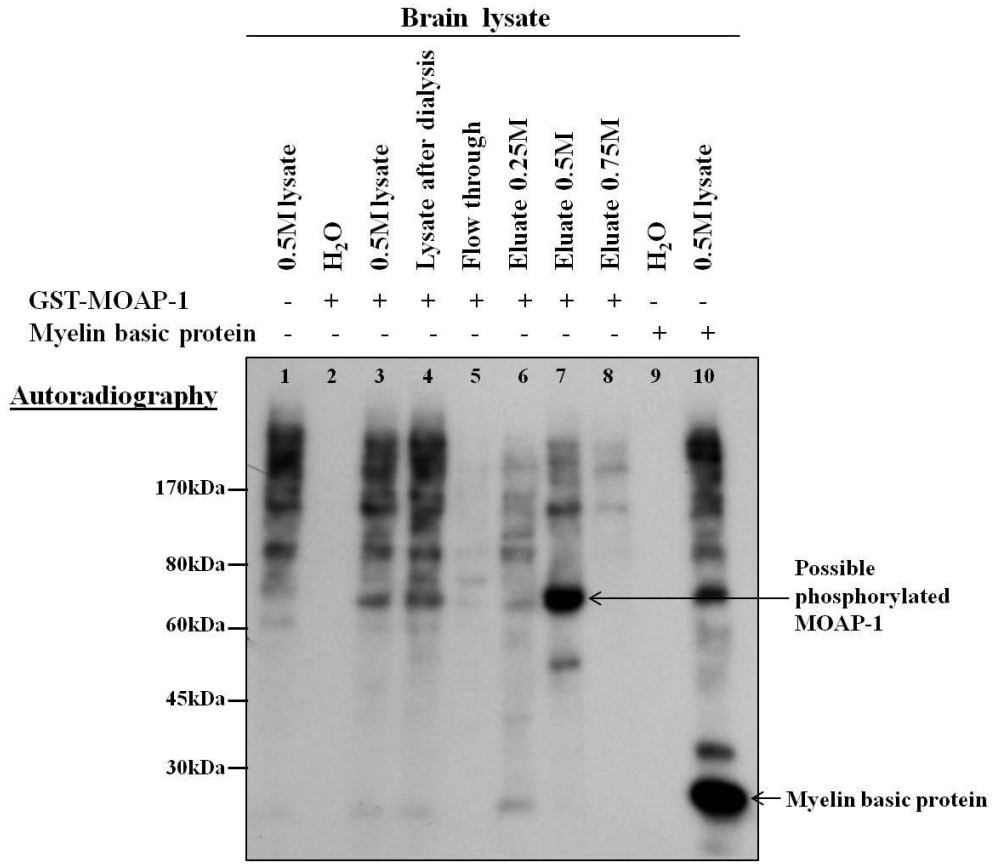

Figure 4.2 Autoradiography showed a band corresponding to the molecular weight of GST-MOAP-1 in the purified 0.5M brain elute reaction. For *in vitro* phosphorylation reaction, recombinant GST-MOAP-1 was incubated with 10 μM [γ-^{32}P] ATP and the respective purified elutes for 30 min at 30 ^{o}C. The reactions were stopped by boiling in 2x SDS-PAGE sample buffer. Samples were separated on SDS-PAGE, stained with GelCode Blue and transferred to PVDF membrane for autoradiography.

Indeed, with a repeat of ion exchange column purification, a stronger signal corresponding to the molecular weight of GST-MOAP-1 was observed in the same elution concentration of 0.5M NaCl (Fig 4.2, lane 7). In addition, a strong signal corresponding to the molecular weight of MBP was observed (lane 10), suggesting that the *in vitro* kinase assay worked as expected. While the *in vitro* kinase assay using [γ-^{32}P] ATP gave preliminary evidence suggesting that recombinant MOAP-1 can be phosphorylated by protein kinases present in the mouse brain, we were unable to confirm that the band

corresponding to the molecular weight of GST-MOAP-1 is indeed GST-MOAP-1. More work would need to be conducted to confirm whether the putative phosphorylated band is MOAP-1 and to identify and characterize the kinase(s) present in the brain that phosphorylate recombinant GST-MOAP-1.

In an attempt to detect phosphorylated forms of endogenous MOAP-1, we explored the possibility if the supplementation of PhosSTOP phosphatase inhibitor cocktail tablet (Roche) in the lysis buffer will allow a visual differentiation from the non-phosphorylation species of MOAP-1 and the detection of phosphorylated forms of MOAP-1 in western blotting. To address this, we harvested HEK293T cells treated with proteasome inhibitor, MG132 and apoptotic stimuli known to up-regulate MOAP-1, Etoposide (ETOP). Protein samples were lyzed in lysis buffer supplemented with and without PhosSTOP phosphatase inhibitor cocktail tablet (Roche). SIRT1, a NAD^+-dependent deacetylase was reported to be a phospho-protein (206) and hence was immuno-blotted as a positive control.

As expected, proteasomal inhibition by MG132 and DNA-damaging apoptotic stimuli, ETOP up-regulated protein levels of endogenous MOAP-1 in HEK293T cells (Fig 4.3A). However, no noticeable difference in protein levels or migration species was observed. In contrast, the immuno-blot for SIRT1 showed a robust up-regulation of its protein levels in the presence of the PhosSTOP phosphatase inhibitor (Fig 4.3B). The data obtained from this experiment was not able to aid the detection of phosphorylated species of endogenous MOAP-1. It may suggest that that phosphorylation mechanism does not play a direct role in regulating the protein stability of endogenous MOAP-1 in HEK293T cells. However, it is premature to conclude that

MOAP-1 is not a phospho-protein. SIRT2, another member in the Sirtuins family of proteins, was also reported to be regulated by phosphorylation (207). However, similar to MOAP-1, no noticeable difference in its protein levels was observed in the presence or absence of PhosSTOP phosphatase inhibitor (Fig 4.3D).

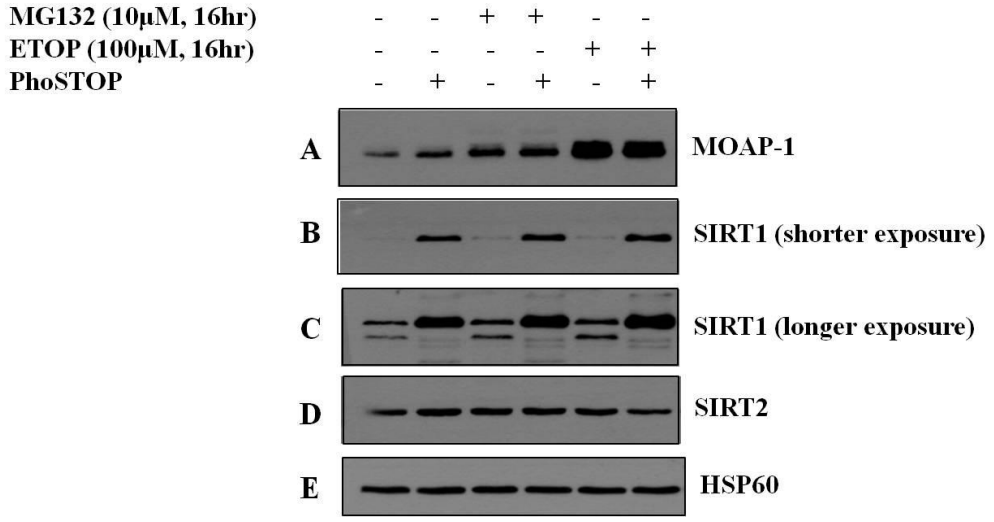

Figure 4.3 Supplementation of PhosSTOP phosphatase inhibitor cocktail tablet in the lysis buffer did not regulate the protein stability of MOAP-1. HEK293T cells were treated with proteasomal inhibitor, 10 μM MG132 and apoptotic stimuli, 100 μM ETOP for 16 hours). The cells were harvested and subjected to SDS-PAGE and western blotting. HSP60 was used as an internal control to demonstrate that equal amount of proteins was loaded in each lane.

4.2.2 Identification and mapping of phosphorylation sites on GST-MOAP-1

In an attempt to identify and map phosphorylation sites on recombinant GST-MOAP-1 by mass spectrometry analysis, we set up a collaboration with Li Rong from Experimental Therapeutics Centre, Biopolis, Singapore. *In vitro* phosphorylation of recombinant GST-MOAP-1 was carried out with the incubation of GST-MOAP-1, brain lysate and ATP in a kinase buffer at 30^{o}C

for 30min. Reactions were stopped by boiling the samples in 2x SDS-PAGE sample buffer. Protein samples were subjected to SDS-PAGE and the gels were subsequently submitted to the collaborator for mass spectrometry analysis.

Three phosphorylation sites were identified in MOAP-1 from these samples. All three serine sites namely S27, S29 and S31 were found in one peptide (KALLIAGI**S**Q**S**C**S**VAEIEEALQAGLAPLGEYR). It is noteworthy however that these three serines are conserved only in chimpanzee human and monkey but not in mouse and rat (Fig 4.4). Though not identified by the mass spectrometry, another residue of interest in the MOAP-1 protein sequence is the conserved ERK phosphorylation motif (S/T)P, S173. (S/T)P motif has been reported to be potential sites of ERK phosphorylation (208). For this ERK conserved motif, it is only conserved in chimpanzee and human. Interestingly, just four amino acids away, we observed a serine that is conserved in all the species examined, S169 (Fig 4.4).

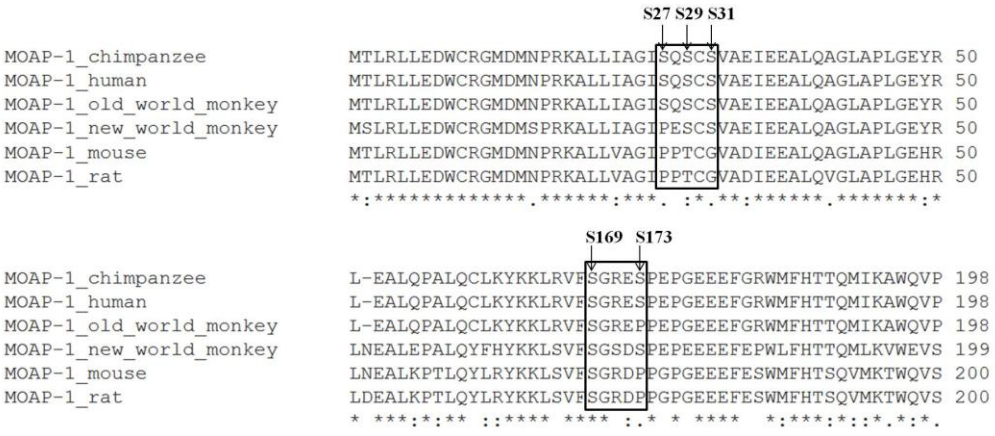

Figure 4.4 Identification of five potential phosphorylation serine residues. Multiple protein sequence alignment was carried using ClustalW2 algorithm.

To evaluate the potential significance of these phosphorylation sites on the protein stability of MOAP-1, we conducted site-directed mutagenesis to generate a series of mutants of human MOAP-1 in which each of the five selected serines was substituted with either alanine or aspartate (Fig 4.5). The alanine substitution prevents phosphorylation at that residue and can mimic constitutively de-phosphorylated serine while the aspartate substitution can mimic constitutively phosphorylated serine.

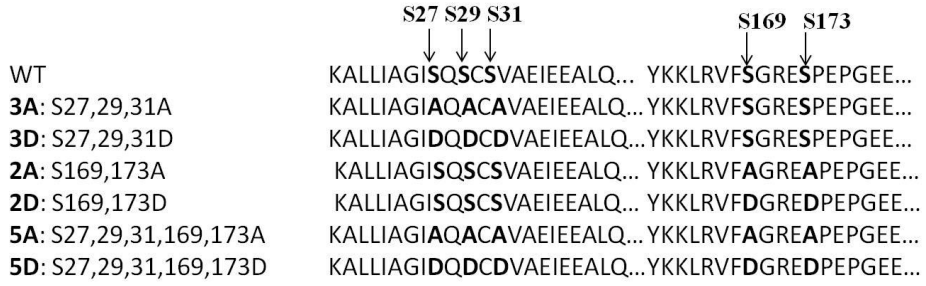

Figure 4.5 MOAP-1 mutants with point mutations at serine residues 27, 29, 31, 169 and 173. Each mutant is listed and differences from the WT sequence are indicated in bold.

Next, we tested the expression of these MOAP-1 mutants. HEK293T cells were used due to its high transfection efficiency. HA-MOAP-1 WT and the respective mutants were transfected in HEK293T cells and harvested after 48 hours. Western blot analysis using HA antibody showed that mutants MOAP-1-3D and MOAP-1-5D have elevated protein expression compared to MOAP-1-WT or the other mutants (Fig 4.6) suggesting that constitutive phosphorylation at serine residues 27, 29 and 31 may be involved in mediating the protein stability of MOAP-1.

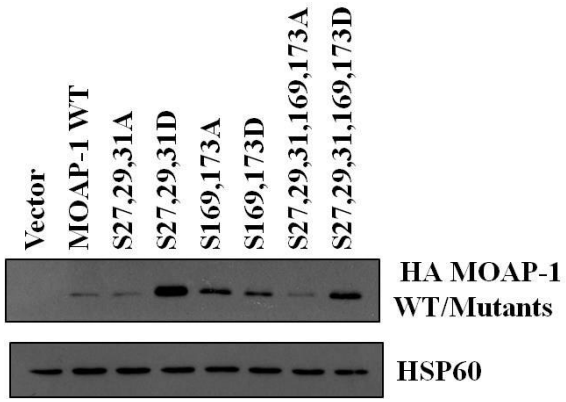

Figure 4.6 Expression of MOAP-1 mutants in HEK293T cells. HEK293T cells were transfected with wild type (WT) and the respective MOAP-1 mutants for 48 hours. Protein samples were harvested, separated on SDS-PAGE gel and analyzed with western blotting. HSP60 was used as an internal control to demonstrate that equal amount of proteins was loaded in each lane.

4.2.3 Characterization of potential phosphorylation sites identified by mass spectrometry analysis

Since it was observed that the MOAP-1-3D mutant has higher protein expression than the other mutants, we hypothesized that phosphorylation at the serine residues 27, 29 and 31 may stabilize MOAP-1 and consequently result in the higher abundance of protein levels observed. This led us to investigate the half-life of the MOAP-1-3D mutant using cycloheximide (CHX) chase assay. MOAP-1-WT, MOAP-1-3D and MOAP-1-2D mutants were transiently transfected in HEK293T cells. CHX, which inhibits de novo protein synthesis, was added to the cells at 24 hours post-transfection. Protein samples were harvested after 5 hours of CHX treatment.

As shown in Fig 4.7, MOAP-1-3D mutant was indeed found to be more stable as compared to MOAP-1-WT (lanes 4 and 5), suggesting that phosphorylation at these serine residues 27, 29 and 31 may aid in stabilizing

MOAP-1, thereby extending its half-life. Notably, the expression of MOAP-1-2D was very weak in this experiment and hence we were not able to conclude if the stability of MOAP-1-2D mutants were affected by constitutive phosphorylation at serine residues 169 and 173 (lane 3). For the CHX chase assay, Mcl-1, a short half-life Bcl-2 family protein, was used as a positive control to ensure that CHX blocked new protein synthesis in these cells while HSP60 was used as a loading control.

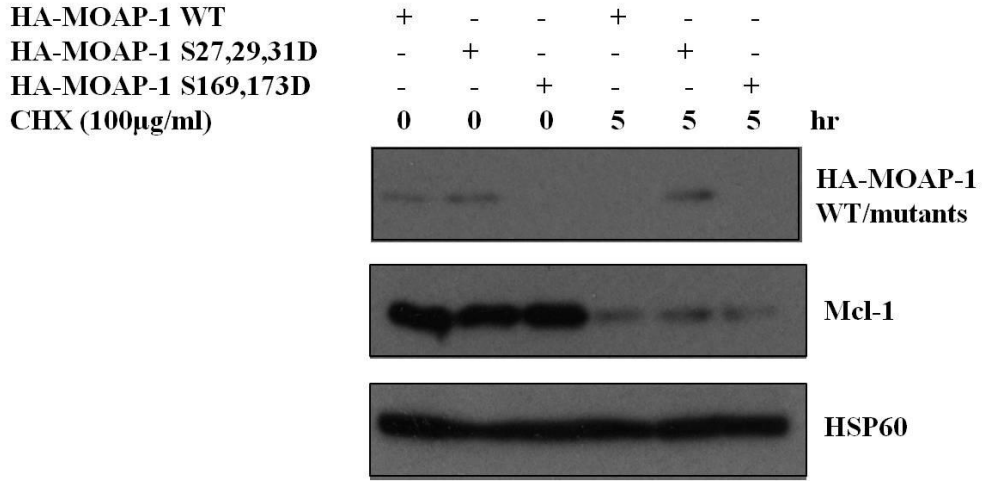

Figure 4.7 Mutation of serine (S) to aspartate (D) at residues 27, 29 and 31 stabilized MOAP-1. HEK293T cells were transfected with HA-MOAP-1-WT, HA-MOAP-1-3D and HA-MOAP-1-2D for 24 hours. 100 µg/ml cycloheximide (CHX) was added to the cells for 5 hours. Protein samples were harvested, separated on SDS-PAGE gel and analyzed with western blotting. Mcl-1 was used as a positive control to demonstrate that the CHX chase assay worked as expected. HSP60 was used as an internal control to demonstrate that equal amount of proteins was loaded in each lane.

The up-regulation of MOAP-1 by apoptotic stimuli has been well documented (67). Next, we investigated whether phosphorylation at serine residues 27, 29, 31, 169 and 173 are required for MOAP-1 up-regulation by apoptotic stimulus, ETOP. Alanine mutants MOAP-1-2A, MOAP-1-3A and MOAP-1-5A were transiently transfected into HEK293T cells along with a

vector control. ETOP was added to the cells at 24 hours post-transfection and were treated for 16 hours. The non-phosphorylatable mutants (MOAP-1-3A, MOAP-1-2A and MOAP-1-5A) were robustly up-regulated by ETOP, suggesting that phosphorylation at these residues may not play a significant role in mediating MOAP-1 stabilization by ETOP (Fig 4.8). Notably, MOAP-1 has a total of 11 serine, 13 threonine and 6 tyrosine residues, all of which (in addition to the five serine residues examined) are potential phosphorylation sites that may be directly involved in the molecular regulation of MOAP-1 under apoptotic treatment by ETOP.

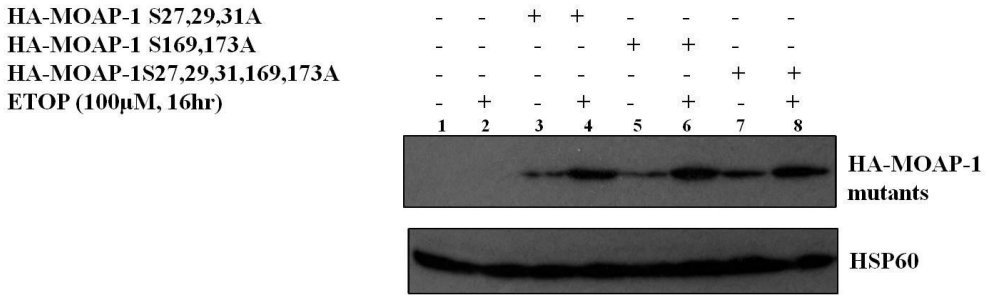

Figure 4.8 Up-regulation of the non-phosphorylatable alanine mutants by apoptotic stimulus, ETOP. HEK293T cells were transfected with HA-MOAP-1-3A, HA-MOAP-1-2A and HA-MOAP-1-5A for 24 hours. 100 μM Etoposide (ETOP) was treated to the cells for 16 hours. Protein samples were harvested, separated on SDS-PAGE gel and analyzed with western blotting. HSP60 was used as an internal control to demonstrate that equal amount of proteins was loaded in each lane.

4.2.4 Inhibition of MAPK and PI3K signaling cascades by chemical-based kinase inhibitors

As mentioned, protein levels of endogenous MOAP-1 is elevated by multiple apoptotic stimuli including TRAIL, THA, ETOP, ultra-violet irradiation and serum withdrawal in multiple mammalian cell-lines such as 293T, HCT116, SY5Y, HepG2, H1299 and HeLa cells (67). Notably, STS is

the only apoptotic stimulus tested that is unable to up-regulate endogenous protein levels of MOAP1 (67). Interestingly, STS is recognized as a wide-spectrum ATP-competitive kinase inhibitor that binds to and inhibit many protein kinases with high affinity (209,210).

4.2.4.1 Inhibition of MAPK signaling cascade

It has been reported that the regulation and commitment of apoptosis involve kinase signaling pathways including, but not limited to, the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) pathways (211-213). The MAPK is a family of serine/threonine kinases that mediate intracellular signal transduction in response to various stimuli (214). To date, major MAPKs include the extracellular signal-regulated kinases (ERK1/2), stress-activated protein kinases [c-Jun NH_2-terminal kinases (JNK)] and p38 mitogen-activated protein kinases. Similarly, the PI3K pathway responds to a variety of extra- and intra-cellular signals that regulate many cellular processes such as apoptosis and cellular proliferation (116).

We hypothesized that the up-regulation of endogenous MOAP-1 by multiple apoptotic stimuli may involve and depend on the MAPK and PI3K signaling pathways. To address this, we blocked the MAPK and PI3K signaling pathways using established chemical-based kinase inhibitors and evaluate if the up-regulation of MOAP-1 by apoptotic stimulus is affected. ETOP is one of the apoptotic stimuli that results in a robust up-regulation of endogenous MOAP-1 in HEK293T cells. Hence, ETOP and HEK293T cells were chosen as the apoptotic stimulus and cell-line to be used in the kinase inhibitors experiments. HEK293T cells were subjected to an hour of pre-

treatment with the selected kinase inhibitors in the presence and absence of ETOP for 16 hours. Endogenous protein level of MOAP-1 was probed to evaluate if the up-regulation by apoptotic stimulus was blocked and HSP60 was used as a loading control. It is noteworthy that the concentration of the kinase inhibitors used was based on concentrations that are widely used and accepted in the literature for inhibition of the respective kinases. In addition, before each harvest, cells were monitored in terms of their morphology and attachment to the petri dishes to ensure that the kinase inhibitors were not causing notable cyto-toxicity. Due to limitations in resources of the antibodies, positive controls such as immuno-blot of the targeted kinase and its downstream kinases were not conducted as a way for confirming the fidelity of the inhibitors used. However, validation of the results with over-expression of the specific kinase(s) will be conducted once preliminary data have shown sufficient evidence that the kinase(s) is/are involved in the molecular regulation of MOAP-1.

SB203580 and DJNK1 are potent inhibitors of the p38 and JNK kinases respectively while PD98059 and U0126 are potent inhibitors of MEK1/2 kinase. SB203580 inhibits the catalytic activity of p38 by competitive binding in the ATP pocket (215) while DJNK1 is a JNK peptide inhibitor that inhibits the interaction between JNK and its substrate (216). At the concentrations tested, inhibition of p38 or JNK did not suppress the up-regulation of endogenous MOAP-1 by ETOP (Fig 4.9 A&B). Although PD98059 and U0126 inactivate MEK1/2 through different mechanisms [PD98059 inhibits MEK1/2 by competitive binding to MEK1/2 thereby preventing the activation of MEK1/2 by its up-stream kinase, Raf (217) while

U0126 directly inhibits MEK1/2 by acting on its catalytic properties (218)], both inhibitors showed significant effect in blocking the up-regulation of endogenous MOAP-1 by ETOP but did not affect basal levels of MOAP-1 (Fig 4.9 C&D). These results suggest that the MEK1/2 (but not p38 or JNK kinases) and/or its downstream targets may be involved in mediating the elevation of endogenous protein level of MOAP-1 under apoptotic condition induced by ETOP.

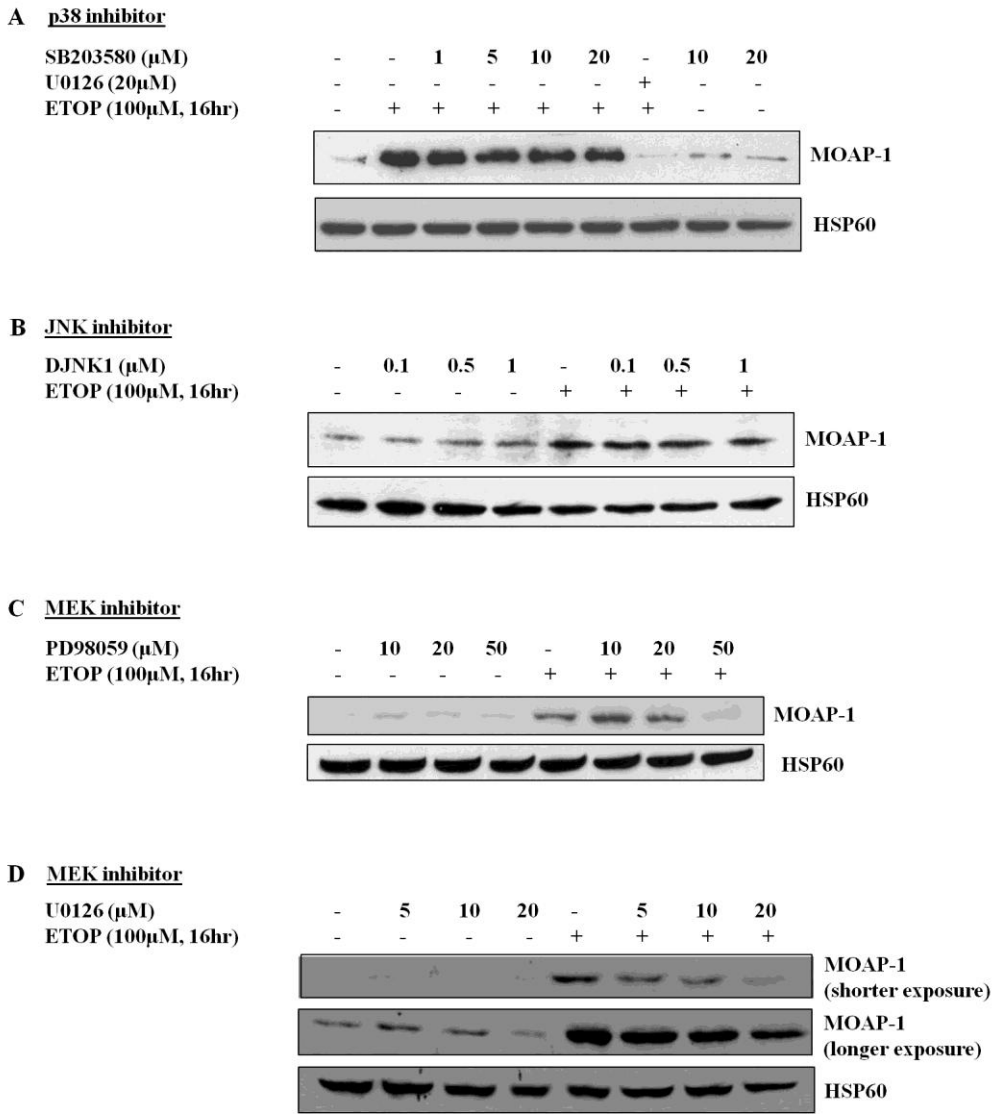

Figure 4.9 Chemical-based inhibition of p38, JNK and MEK signaling cascades. HEK293T cells were pre-treated with the respective kinase inhibitors at the indicated concentrations for 1 hour before being subjected to 100 μM ETOP treatment for 16 hours. The cells were harvested and proteins were resolved by SDS-PAGE and analyzed with western blotting. HSP60 was used as an internal control to demonstrate that equal amount of proteins was loaded in each lane. **(A)** Inhibition of p38 by SB203580 did not block the elevation of MOAP-1 protein level by apoptotic stimulus, ETOP. **(B)** Inhibition of JNK by DNK1 did not suppress the up-regulation of endogenous MOAP-1 by ETOP. **(C & D)** Inhibition of MEK1/2 by PD98059 and U0126 respectively blocked the up-regulation of MOAP-1 by apoptotic stimulus, ETOP.

4.2.4.2 Inhibition of PI3K signaling cascade

Wortmannin and LY294002 are potent and commonly used inhibitors of PI3Ks. Wortmannin is a potent pan-specific inhibitor that occupies the ATP-binding site of p110 by forming a covalent bond between C20 of the Wortmannin furan ring and K802 of p100 of PI3K (219). However, Wortmannin has a half-life of only a few minutes in serum due to its highly reactive C20 position (220). LY294002, a reversible synthetic compound, makes a key hydrogen bond between the morpholino oxygen in the compound and the backbone amide of V882 of p110, mimicking the interaction made by adenine of ATP (221,222). PI3K inhibition by both Wortmannin and LY294002 showed significant effect in blocking the up-regulation of endogenous protein level of MOAP-1 by ETOP but did not affect basal levels of MOAP-1 (Fig 4.10 A&B) suggesting that the PI3K signaling pathway may potentially be also involved in mediating the molecular regulation of MOAP-1 under apoptotic condition.

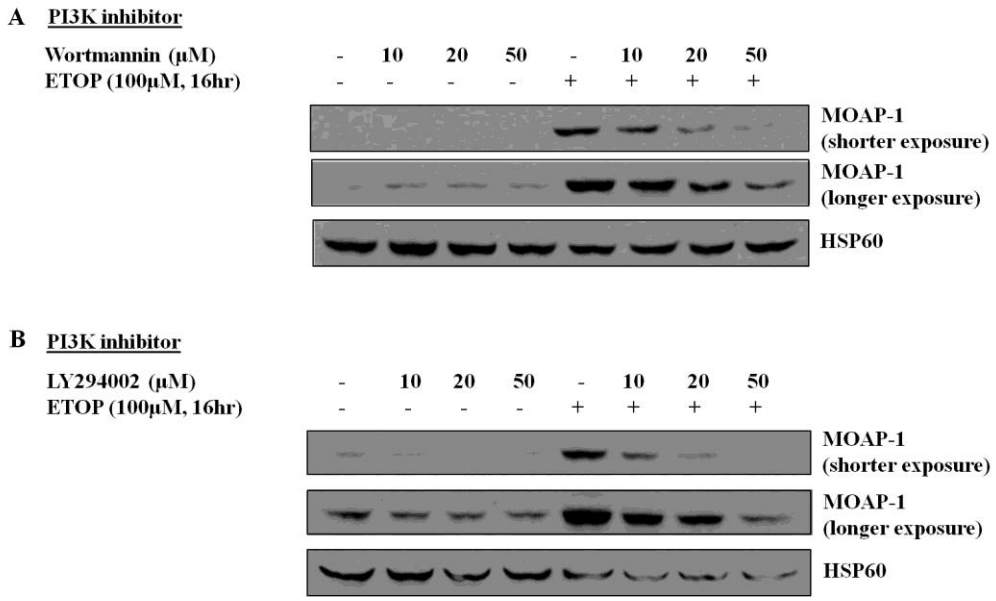

Figure 4.10 Inhibition of PI3K signaling cascade by Wortmannin (A) and LY294002 (B) suppressed the up-regulation of endogenous protein level of MOAP-1 by apoptotic stimulus, ETOP. HEK293T cells were pre-treated with Wortmannin and LY294002 at the indicated concentrations for 1 hour before being subjected to 100 μM ETOP treatment for 16 hours. The cells were harvested and proteins were resolved by SDS-PAGE and analyzed with western blotting. HSP60 was used as an internal control to demonstrate that equal amount of proteins was loaded in each lane.

mTORC1 is a serine-threonine kinase that is a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family of kinases. mTORC1 exists as a complex consisting of mTOR with raptor, LST8 and AKTS1. Rapamycin is a specific and potent inhibitor of mTORC1 which inhibits mTORC1 by dissociating raptor from the complex (223). Since the inhibition of PI3K signaling pathway showed significant suppression of the up-regulation of endogenous MOAP-1 by ETOP and mTORC1 is a key downstream target of the PI3K signaling pathway, we asked if inhibition of mTORC1 by Rapamycin will give similar result as that observed by Wortmannin and LY294002 (Fig 4.10). Indeed, inhibition of mTORC1 by Rapamycin showed significant suppression of endogenous MOAP-1 elevation by ETOP (Fig 4.11).

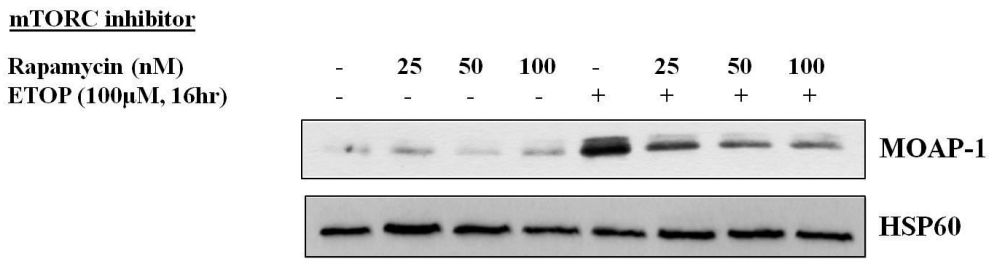

Figure 4.11 Suppression of mTORC by Rapaymycin blocked endogenous MOAP-1 up-regulation by apoptotic stimulus, ETOP. HEK293T cells were pre-treated with Rapamycin at the indicated concentrations for 1 hour before being subjected to 100μM ETOP treatment for 16 hours. The cells were harvested and proteins were resolved by SDS-PAGE and analyzed with western blotting. HSP60 was used as an internal control to demonstrate that equal amount of proteins was loaded in each lane.

Although Wortmannin and LY294002 are widely employed as PI3K inhibitors, it was reported by several studies that they both have significant limitations and hence are not ideal inhibitors for PI3Ks (224). While Wortmannin is a reactive electrophilic and hence unstable compound, LY294002 is a weak inhibitor with only micro-molar potency. Furthermore, both compounds display significant off-target effects on other kinases (224-229). PI-103 is a relatively new potent, cell-permeable, ATP-competitive inhibitor that targets both PI3K and mTORC1 (230-233). As a multi-target inhibitor, PI-103 targets the catalytic domain of PI3K and mTORC1, delivering a powerful inhibition of the PI3K and mTORC1 pathways (228). It was reported that PI-103 exhibited advantages over Wortmannin and LY294002 in terms of its excellent potency, selectivity for PI3Ks and improved stability (224). Thus, we asked if suppression of endogenous MOAP-1 up-regulation by ETOP will be observed under double inhibition of both PI3K and mTORC1 by PI-103. On contrary, inhibition of PI3K and mTORC1 by PI-103 did not suppress the elevation of endogenous MOAP-1

by apoptotic stimulus, ETOP (Fig 4.12). This contradicts the data obtained from Wortmannin and LY294002 in Fig 4.10.

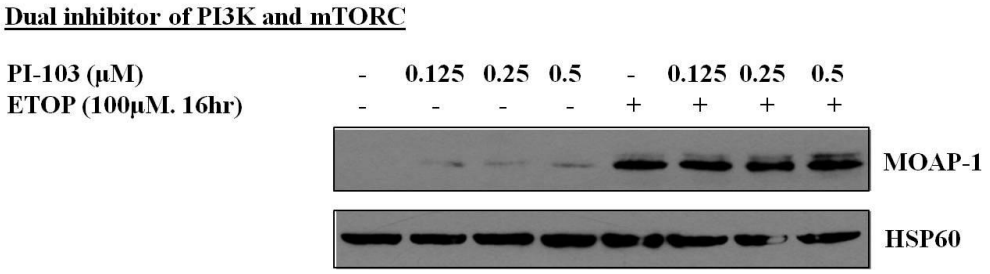

Figure 4.12 Dual inhibition of PI3K and mTORC did not suppress the up-regulation of MOAP-1 by apoptotic stimulus, ETOP. HEK293T cells were pre-treated with PI-103 at the indicated concentrations for 1 hour before being subjected to 100 μM ETOP treatment for 16 hours. The cells were harvested and proteins were resolved by SDS-PAGE and analyzed with western blotting. HSP60 was used as an internal control to demonstrate that equal amount of proteins was loaded in each lane.

Taken together, it seems inconclusive whether the inhibition of PI3K is able to block the up-regulation of endogenous MOAP-1 under the treatment of ETOP. Although Rapamycin is recognized as an exquisitely specific inhibitor of mTORC1 (234), it may be premature to conclude that mTORC signaling pathway is directly involved in regulating the protein stability of MOAP-1 under ETOP treatment. Remarkably, it was reported that Wortmannin can inhibit the chemotactic peptide-induced activation of MEK2, the predominant form in human neutrophils (235). In this scenario, it is conceivable that perhaps Wortmannin may inhibit the activation of MEK2 by ETOP treatment, thereby suppressing the up-regulation of MOAP-1 by ETOP. On contrary, there is no article found to date, that reports the inhibition on the MEK/MAPK signaling pathway by PI3K inhibitor, LY294002. Hence, we decided to focus our study on the MEK/MAPK signaling cascade, since inhibition of MEK1/2 by both PD98059 and U0126 have consistently suppressed the elevation of

endogenous MOAP-1 by ETOP, suggesting that MEK and/or its downstream targets are likely to be involved in mediating the up-regulation of endogenous MOAP-1 under DNA-damaging apoptotic stimulus, ETOP in HEK293T cells.

4.2.5 ETOP activates the ERK/MAPK signaling cascade

It was reported that ERK is activated by DNA damaging signals such as ETOP which interferes with the function of DNA topoisomerase II leading to the breakage of double-strand DNA (236,237). We asked if such phenomenon will be observed in HEK293T cells under ETOP treatment. To address this, we treated 100 μM ETOP to HEK293T cells for the indicated duration. As shown in Fig 4.13, ERK activation was rapidly observed under the treatment of ETOP.

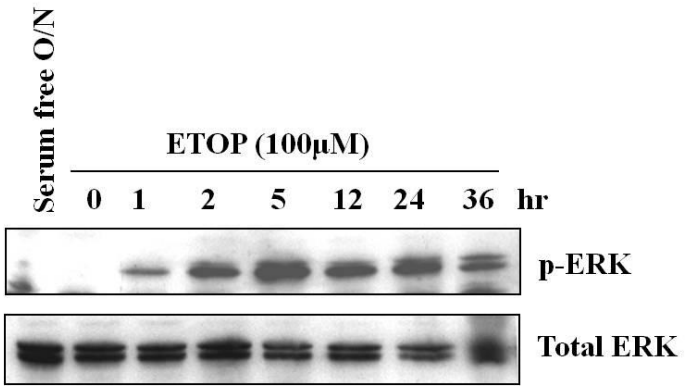

Figure 4.13 ETOP activates ERK in HEK293T cells. HEK293T cells were starved with serum-free DMEM for 16 hours overnight. Cells were then subjected to 100 μM ETOP treatment for the indicated duration. The cells were harvested and subjected to SDS-PAGE and western blotting. Total ERK was used as an internal control to demonstrate that equal amount of proteins was loaded in each lane.

4.2.6 MEKΔ is involved in regulating the protein stability of MOAP-1

Results obtained from the inhibition of MEK1/2 by PD98059 and U0126 suggest that molecular targets in the MAPK signaling cascade may be

involved in mediating the up-regulation of endogenous MOAP-1 under ETOP treatment. Hence, we asked if over-expression of constitutively active S218, 222D MEK1 (referred to as MEK1Δ in this report) (238), a key regulatory kinase in the MAPK signaling cascade, will regulate the protein level of MOAP-1 under co-expression and single expression conditions. For the co-expression experiment, HEK293T cells were transiently transfected with expression vectors encoding FLAG-MOAP-1 and HA-MEK1Δ. While for the single expression experiment, HEK293T cells were transiently transfected with HA-MEK1Δ along with a vector control and harvested after 48 hours. As illustrated in Fig 4.14, MEK1Δ resulted in a robust up-regulation of FLAG-MOAP-1 (A) and endogenous MOAP-1 (B), suggesting the possible involvement of MEK1 in mediating the protein stability of MOAP-1. In addition, over-expression of MEK1Δ also induced the activation of ERK, a direct substrate of MEK (119) (Fig 4.14B).

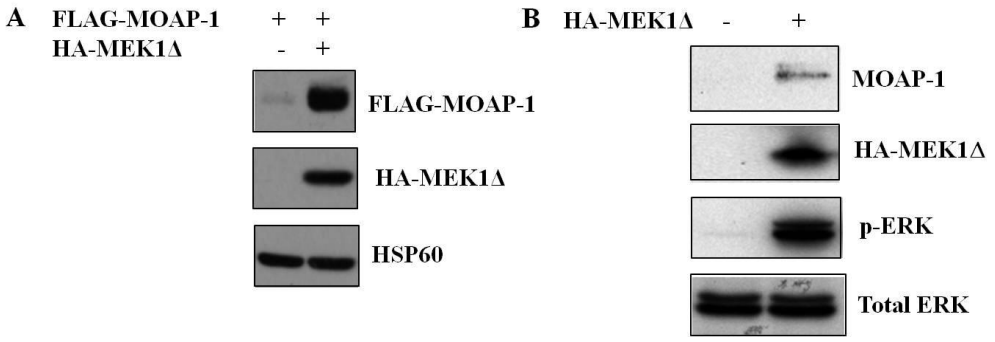

Figure 4.14 MEK1Δ up-regulates exogenous (A) and endogenous (B) protein level of MOAP-1 in HEK293T cells. (A) For co-expression experiment, HEK293T cells were transfected with HA-MEK1Δ and FLAG-MOAP-1 for 48 hours. **(B)** HEK293T cells were transfected with HA-MEK1Δ along with a vector control for 48 hours. The cells were harvested and subjected to SDS-PAGE and western blotting analysis. HSP60 (in A) and total ERK (in B) were used as internal control to demonstrate that equal amount of proteins was loaded in each lane.

4.2.7 MEKΔ interacts with MOAP-1

Next, we asked if a physical interaction between MOAP-1 and MEK1Δ can be observed. To address this, HEK293T cells were transiently transfected with expression vectors encoding MYC-MOAP-1 and HA-MEK1Δ for 48 hours. The cells were then harvested and equal amounts of total cell lysates were subjected to immuno-precipitation (IP) with either HA or MYC antibodies. As shown in Fig 4.15, MOAP-1 was found to interact with MEK1Δ under co-expression condition in HEK293T cells suggesting that MEK1Δ may play a direct role in mediating the protein stability of MOAP-1.

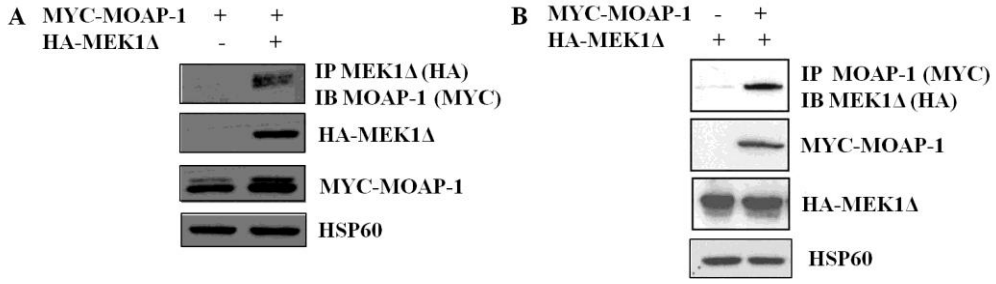

Figure 4.15 MEK1Δ interacts with MOAP-1. HEK293T cells were transiently transfected with MYC-MOAP-1 and HA-MEK1Δ for 48 hours. The cells were harvested and equal amounts of total cell lysates were immuno-precipitated with anti-HA (in A) and anti-MYC (in B). Immuno-precipitates were immuno-blotted with anti-MYC (in A) and anti-HA (in B). The cell lysates and immuno-precipitates were resolved by SDS-PAGE and analyzed by western blotting.

4.2.8 MEKΔ stabilizes MOAP-1 by extending its half-life

Next, we asked if MEK1Δ up-regulates MOAP-1 by influencing the protein stability of MOAP-1. Using the cycloheximide (CHX) chase assay, we investigated the effect of MEK1Δ on the half-life of exogenous and endogenous MOAP-1 in HEK293T cells. For the study on exogenous MOAP-1, 100μg/ml CHX was added to HEK293T cells transiently over-expressing both FLAG-MOAP-1 and HA-MEK1Δ. Steady-state protein levels of FLAG-

MOAP-1 were assessed at 1, 3 and 5 hours after CHX treatment. As for the study on endogenous MOAP-1, HEK293T cells were transfected with HA-MEK1Δ along with a control vector. At 24 hours post-transfection, 100 μg/ml CHX was added to the cells. Steady-state protein levels of endogenous MOAP-1 were monitored at 0.5, 1 and 2 hours after CHX treatment.

As shown in Fig 4.16A, in the presence of MEK1Δ, FLAG-MOAP-1 was up-regulated and stabilized even at 5 hours of CHX treatment, suggesting that MEK1Δ up-regulates exogenous MOAP-1 via stabilization of the MOAP-1 protein. Mcl-1, a short half-life Bcl-2 family protein, was used as a positive control to ensure that CHX blocked new protein synthesis in these cells. Similar results on the protein turn-over of endogenous MOAP-1 were obtained from single over-expression of MEK1Δ. As demonstrated in Fig 4.16B, in the presence of MEK1Δ, endogenous MOAP-1 was stabilized. Taken together, the results suggest that MEK1Δ may have a direct role in modulating protein stability of MOAP-1 by extending its half-life of MOAP-1.

Notably, even though the CHX chase assay is commonly used in assaying protein stability, there are limitations with this assay. CHX inhibits the *de novo* protein synthesis of all proteins in the cells treated. This would imply that apart from MOAP-1, all other proteins including positive or negative regulators of MOAP-1 with relatively short half-life will be rapidly degraded. For instance, degradation of MOAP-1 may be reduced if the E3 ubiquitin ligase of MOAP-1 is also rapidly degraded due to the CHX treatment. Indeed, many E3 ubiquitin ligases including MDM2 have the ability to promote self-ubiquitination, resulting in their rapid degradation and short half-lives (239).

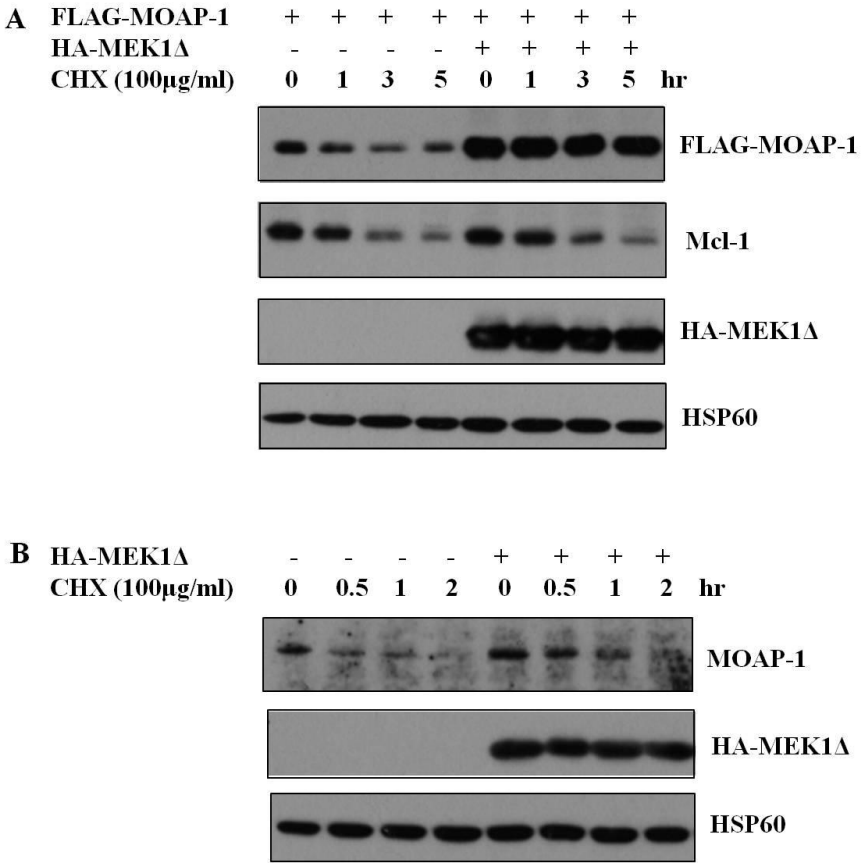

Figure 4.16 MEK1Δ stabilizes exogenous (A) and endogenous (B) MOAP-1 by extending its half-life. (A) HEK293T cells were transiently transfected with HA-MEK1Δ and FLAG-MOAP-1. At 24 hours post-transfection, the cells were treated with 100 μg/ml cycloheximide (CHX) for the indicated duration. After treatment, the cells were harvested and subjected to SDS-PAGE and western blotting analysis. Mcl-1 was used as a positive control to demonstrate that the CHX assay worked as expected. HSP60 was used as an internal control to demonstrate that equal amount of proteins was loaded in each lane. **(B)** HEK293T cells were transfected with HA MEK1Δ for 24 hours. The cells were treated with 100 μg/ml CHX for the indicated duration. After treatment, the cells were harvested and subjected to SDS-PAGE and western blotting analysis. HSP60 was used as an internal control to demonstrate that equal amount of proteins was loaded in each lane.

Furthermore, it was observed that exogenous MOAP-1 is more stable than its endogenous form as the former requires up to 5 hours of CHX treatment for visible degradation (Fig 4.16). One possible explanation could be the difference in cellular localization between endogenous and exogenous MOAP-1. While endogenous MOAP-1 has been shown to be enriched in the

mitochondrial fraction (66), exogenous MOAP-1 was observed to be localized in the cytoplasm by confocal microscopy analysis (refer to Chapter 5 Fig 5.2). This may suggest that the degradation of MOAP-1 via the ubiquitin proteasome system may require MOAP-1 to be localized near or in the mitochondrial and hence may suggest the involvement of mitochondrial E3 ubiquitin ligase(s) for the degradation of MOAP-1.

4.2.9 Alanine mutations at serine residues 27, 29 and 31 abolished MEK1Δ-mediated stabilization of MOAP-1

Previously, we showed that the mutation of serine to aspartate at residues 27, 29 and 31 resulted in the protein stability of MOAP-1 (Fig 4.7). Next, we asked if these serine residues are required for the stabilization of MOAP-1 by MEK1Δ. To do this, HEK293T cells were transiently transfected with HA-MOAP-1-WT or HA-MOAP-1 alanine mutant (HA-MOAP-1-3A) and HA-MEK1Δ for 48 hours. As shown in Fig 4.17, mutation of serine to alanine at residues 27, 29 and 31 abolished the up-regulation of MOAP-1 by MEK1Δ, suggesting that phosphorylation at these residues may be required for the up-regulation and stabilization of MOAP-1 by MEK1Δ.

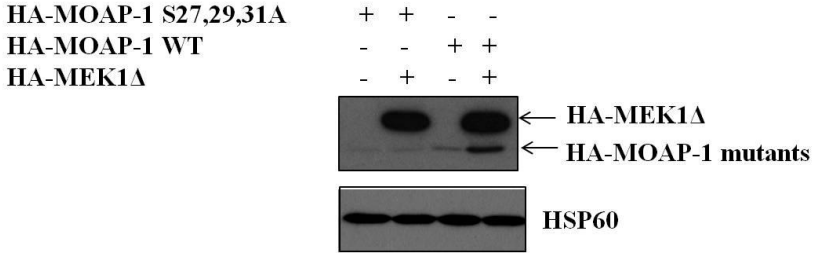

Figure 4.17 Mutation of serine (S) to alanine (A) abolished the stabilization of MOAP-1 by MEK1Δ. HEK293T cells were transiently transfected with HA-MEK1Δ, HA-MOAP-1-3A mutant and HA-MOAP-1-WT for 48 hours. Cells were harvested and subjected to SDS-PAGE and analyzed by western blotting. HSP60 was used as a loading control to demonstrate that equal amounts of cell lysates was loaded into each lane.

4.2.10 MEK1Δ enhances susceptibility of HCT116 cells to TRAIL-induced Bax-activation

Knock-down analysis of MOAP-1 has provided evidence to support an effector role for MOAP-1 in facilitating Bax signaling in mitochondria (66). Furthermore, stable over-expression of MOAP-1 in mammalian cells resulted in sensitization of cells to apoptotic stimuli such as THA and TRAIL (67). Isolated mitochondria from these cells were also more sensitive to the cytochrome c releasing effect of recombinant Bax (67). Since MEK1Δ is able to stabilize MOAP-1, we asked whether it may have an effect on promoting Bax signaling similar to over-expression of MOAP-1. In agreement with this, we investigated the effect of over-expression of MEK1Δ on TRAIL-induced Bax-activation in HCT116 cells. To do this, HCT116 cells were transiently transfected with HA-MEK1Δ along with a control vector for 24 hours. The indicated concentration of TRAIL was then added to the cells for 5 hours. The cells were harvested and equal amounts of total cell lysates were immuno-precipitated with conformation-specific anti-Bax antibody, N20 which recognizes an epitope in the N-terminal domain of Bax that is exposed only after Bax acquires a membrane-insertion competent conformation (240).

As shown in Fig 4.18, TRAIL-induced Bax activation was observed at a lower concentration of TRAIL treatment in MEK1Δ-over-expressing HCT116 cells as compared vector control cells. In addition, cleaved caspase 7, a classical apoptotic marker, was also detected at lower concentration of TRAIL treatment in the MEK1Δ-over-expressing cells. Taken together, these observations suggested that elevated levels of MOAP-1 caused by the

stabilization effect of MEK1Δ could result in enhanced Bax-activation and hence increased sensitivity to apoptotic stimuli.

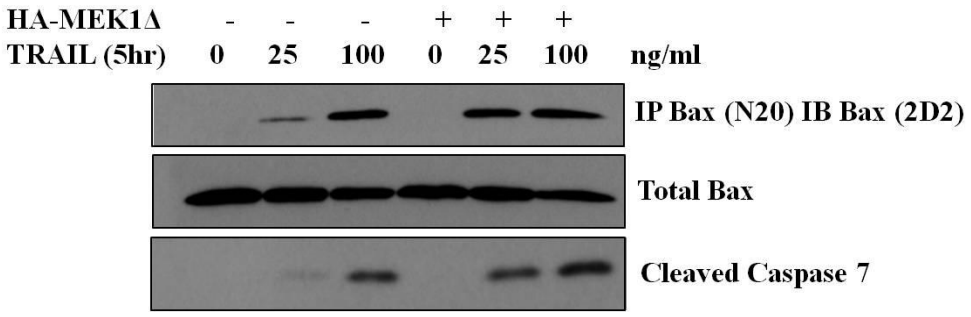

Figure 4.18 MEK1Δ sensitizes HCT116 cells to TRAIL-induced Bax activation and apoptosis. HCT116 cells were transiently transfected with HA-MEK1Δ along with a control vector for 24 hours. The indicated concentration of TRAIL was added to the cells for 5 hours. Extracts were prepared in 2% CHAPS lysis buffer and equal amounts of total cell lysates were immuno-precipitated with the conformation specific anti-Bax antibody, N20 which detects the activated form of Bax. Cell lysates and the immuno-precipitates were resolved by SDS-PAGE and analyzed by western blotting.

4.3 Discussion

Phosphorylation is a major post-translational modification (PTM) mechanism that regulates the function of many Bcl-2 family proteins (69). Reversible phosphorylation and dephosphorylation cascades modulated by kinases and phosphatases are two of the main mechanisms that directly regulate the function and conformation of both pro-survival and pro-apoptotic members of the Bcl-2 protein family (69). It is widely believed that for some proteins, protein phosphorylation plays a critical role in mediating protein ubiquitylation and hence determines whether a protein is targeted for degradation by the UPS (241). Thus, the phosphorylation status of a protein could either inhibit or promote protein ubiquitylation leading to either protein accumulation or degradation.

Data from the chemical-based kinase inhibition of the MAPK and PI3K signaling pathways prompted us to investigate the involvement of MAPK signaling pathway in mediating the protein stability of MOAP-1. Remarkably, MEK1Δ was found to interact with and stabilize MOAP-1 by extending its half-life (Fig 4.14 – 4.16). Furthermore, over-expression of MEK1Δ was shown to sensitize HCT116 cells to TRAIL-induced Bax activation and TRAIL-mediated apoptosis (Fig 4.17), suggesting that MEK1 may possibly be a potential target to modulate the sensitivity of cancer cells to chemotherapeutics through the modulation of MOAP-1 protein stability.

Interestingly, substitution of serine (S) to aspartate (D) at residues 27, 29 and 31 stabilized MOAP-1 as demonstrated in the cycloheximide (CHX) chase assay (Fig 4.7) suggesting the potential involvement of direct phosphorylation at these sites in mediating the protein stability of MOAP-1. Furthermore, alanine substitution of serine residues at 27, 29 and 31 abolished the up-regulation of MOAP-1 by MEK1Δ (Fig 4.18), suggesting that phosphorylation at these sites may be required for the stabilization effect of MOAP-1 by MEK1Δ. Although *in vivo* interaction between MOAP-1 and MEK1Δ was detected under co-expression condition (Fig 4.15), it remains unclear whether MOAP-1 could be a direct substrate of MEK1Δ or its downstream targets. Furthermore, attempts to detect interaction between MEK1Δ and endogenous MOAP-1 were not successful (data not shown). More importantly, the substrate specificity of MEK1 (242,243) makes it unlikely that MEK1 itself functions as a MOAP-1 kinase. As such, it seems more conceivable that ERK1 and/or ERK2 may phosphorylate MOAP-1 directly or through activation of other downstream kinases. To date, direct

phosphorylation of Bcl-2 family proteins by ERK or its downstream targets has been reported in many investigations. For instance, it was documented that ERK has the capacity to phosphorylate and inactivate anti-apoptotic activity of Bcl-2 and thus acts as regulator of the induction of apoptosis (244). Similarly, ERK1/2 can also directly phosphorylate and activate ribosomal s6 kinase, p90rsk which in turn phosphorylates and inactivates pro-apoptotic Bad in the mitochondria, resulting in cellular protection (245).

Results showed in this study have demonstrated that constitutive phosphorylation of MOAP-1 at serines 27, 29 and 31 mediates the protein stability of MOAP-1 and are required for MEK1Δ-mediated stabilization of MOAP-1. It is plausible that activation of the MAPK signaling cascade by DNA damaging apoptotic stimulus, ETOP resulted in the phosphorylation of MOAP-1 which could have result in a change in protein conformation that subsequently deters the E3 ubiquitin ligase from recognizing and targeting MOAP-1 for degradation through the UPS. In addition, a second possibility is that activated ERK1/2 or its downstream targets may inactivate or cause the degradation of the E3 ubiquitin ligase of MOAP-1 thereby resulting in reduced ubiquitination of MOAP-1 and consequently its degradation by the UPS.

Although the kinase of MOAP-1 has not yet been identified, this study provides further insight to enhance our understanding of the mechanisms that serve to regulate MOAP-1 and its role as a potential effector for MAPK-mediated apoptosis.

CHAPTER 5

Identification of Sirtuins as a novel interactors and potential regulators of MOAP-1

5.1 INTRODUCTION

Sirtuins belong to the class III protein deacetylases family, which are the only histone deacetylases (HDACs) that require NAD for their enzymatic activity (246). NAD is a vital co-factor for the electron transport chain and is also involved in many enzymatic reactions (247). Due to their characteristic NAD requirement for their enzymatic reaction, the activity of sirtuins has been directly associated with the metabolic state in the cell. In the last decade, sirtuins have emerged as a crucial family of enzymes in the regulation of eukaryotic metabolism. In mammalians, sirtuins control whole body metabolic homeostasis and are postulated as promising targets for multiple patho-physiological states, including cancer, neuro-degeneration and cardiovascular disease (248,249). An emerging trend from these studies is that sirtuins may have critical roles in the regulation of apoptosis. Interestingly, some members of the sirtuin family have been shown to be localized in mitochondria. Ubiquitination is thus far the only PTM of MOAP-1 established and the involvement of deacetylases in the molecular regulation of MOAP-1 has not been reported previously. Hence, we asked if sirtuins may be involved in mediating the protein stability of MOAP-1.

5.2 RESULTS

5.2.1 Subcellular co-localization profiles of MOAP-1 and SIRT1-5

To evaluate the potential interaction between MOAP-1 and sirtuins, we conducted a preliminary evaluation on the subcellular co-localization between MOAP-1 and sirtuins. SIRT1-5 are included in this scope of study as SIRT1-5 are localized either in the cytosol or in mitochondria in which MOAP-1 has been previously reported to be present under physiological conditions. SIRT6 and SIRT7 are excluded due to their exclusive localization in the nucleus and nucleolus respectively. HeLa cells were transiently transfected with plasmids encoding GFP-MOAP-1 and FLAG-SIRT1-5 for 48 hours. Transfected cells were then analyzed by in-direct immuno-fluorescence analysis for GFP and FLAG –antibodies.

Though endogenous MOAP-1 has been reported to be enriched in the mitochondria fraction (66), over-expressed GFP-MOAP-1 was observed to localize diffusely in the cytosol (Fig 5.1). As illustrated in Fig 5.1, SIRT1 is to be localized in both the nuclear and cytosol compartments. However, in our confocal microscopy analysis of FLAG-SIRT1, it was revealed that SIRT1 is mainly localized in the cytosol (Fig 5.1A). Notably, it was reported that SIRT1 is localized in the nucleus of normal cells, but is predominantly localized in the cytosol of the cancer/transformed cells (250). Since HeLa, a human cervical cancer cell-line, was used in this confocal microscopy analysis, the finding in the report may aid in explaining why cytosolic SIRT1 was observed in our study. In agreement with the literature, our confocal microscopy analysis demonstrated that SIRT2 is primarily localized in the cytosol (Fig

5.1B). Similarly, for mitochondrial proteins SIRT3 and SIRT4, they are, as reported, primarily localized in the mitochondria, revealing the mitochondrial structure and network of the transfected cell (Fig 5.1C-D). On contrary, while SIRT5 is reported to be a mitochondrial protein (176), our confocal microscopy analysis did not illustrate such findings. Instead, both cytosol and nuclear localization of SIRT5 were observed (Fig 5.1E). Notably, it was reported by Pfister *et al*. that SIRT5 is generally localized to both the nucleus and cytosol while mitochondrial localization of SIRT5 is associated with apoptosis (251).

An overlay of the confocal images for GFP-MOAP-1 and FLAG-SIRT1-5 revealed that SIRT2 and SIRT5 showed strong co-localization with MOAP-1 (depicted by the shades of yellow in the overlay images as shown in Fig 5.1B and Fig 5.1E respectively). Zoomed-in images of strong co-localization between MOAP-1 and SIRT2/5 are illustrated in Fig 5.2. Notably, it was difficult to identify cells which had the expression of both GFP-MOAP-1 and SIRT proteins especially for SIRT1 and SIRT4. It may be due to weaker expression of the SIRT1/4 plasmids. In addition, it would be interesting to evaluate if over-expression of SIRT1/4 destabilizes MOAP-1 thereby resulting in MOAP-1 degradation and elimination from the cell.

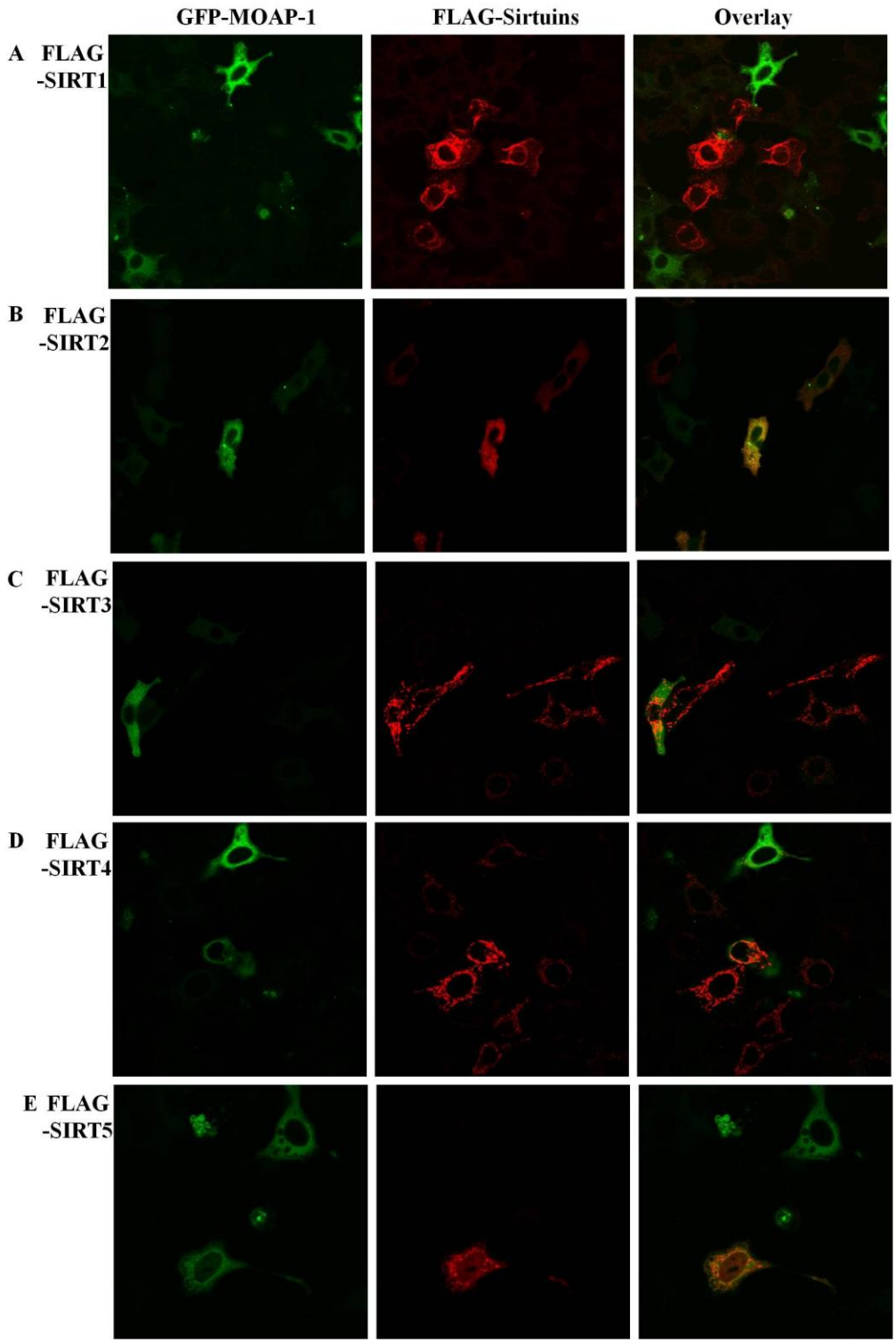

Figure 5.1 Subcellular co-localization of MOAP-1 and SIRT1-5 (depicted in panels A-E respectively) in HeLa cells. Immuno-fluorescent images from confocal microscopy analysis in HeLa cells transiently transfected with plasmids encoding GFP-MOAP-1 and FLAG-SIRT1-5. Transfected cells were then analyzed by immuno-fluorescence analysis for GFP and FLAG-antibody conjugated with the red Cy3 dye.

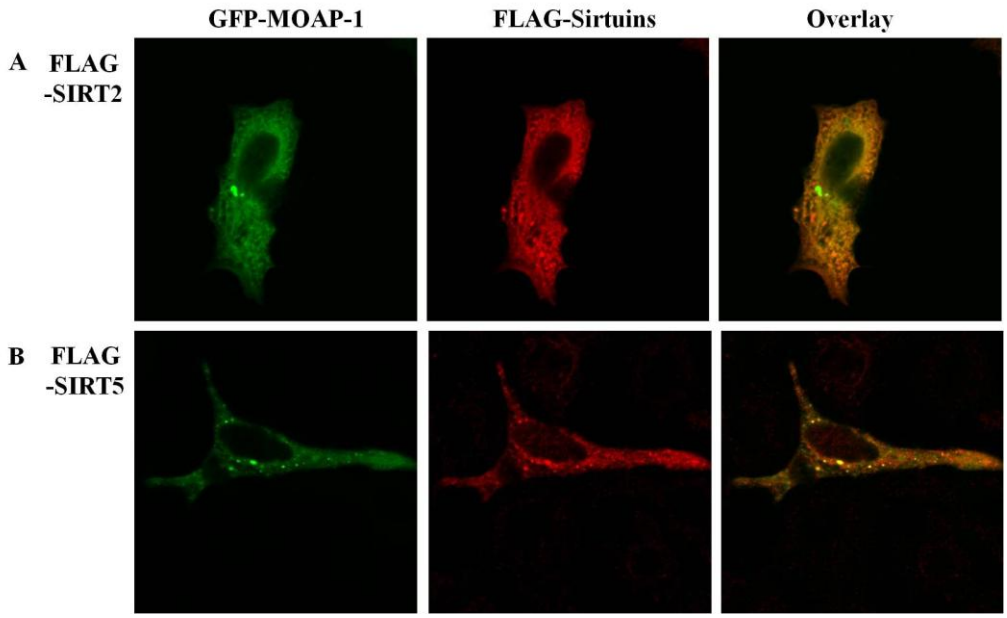

Figure 5.2 Single cell confocal images of MOAP-1 and SIRT2/5 (depicted in panels A and B respectively. Immuno-fluorescent images from confocal microscopy analysis in HeLa cells transiently transfected with plasmids encoding GFP-MOAP-1 and FLAG-SIRT1-5. Transfected cells were then analyzed by immuno-fluorescence analysis for GFP and FLAG-antibody conjugated with the red Cy3 dye.

5.2.2 MOAP-1 interacts with SIRT2 and SIRT5

Although confocal microscopy analysis provided preliminary evidence to support that MOAP-1, SIRT2 and SIRT5 are present in the same subcellular compartment, protein-protein interaction between MOAP-1 and SIRT1-5. To address this, co-immuno-precipitation was conducted to determine interaction between endogenous MOAP-1 and SIRT1-5 in HEK293T cells. HEK293T cells were used due to its high transfection efficiency. As illustrated in Fig 5.3, SIRT2 and SIRT5 interacted with endogenous MOAP-1 in HEK293T cells suggesting that SIRT2 and SIRT5 may be directly involved in mediating the protein abundance or function of MOAP-1. In the same context, MOAP-1 may also be directly involved in modulating the protein stability or activity of

SIRT2 and SIRT5. Additionally, MOAP-1 may serve as a binding protein which is required for SIRT2 and SIRT5 to function properly.

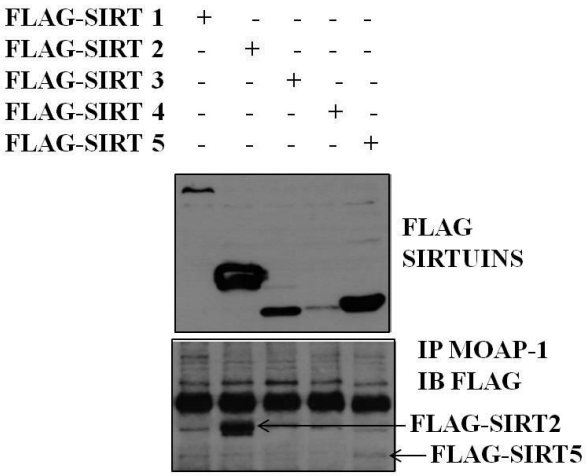

Figure 5.3 Endogenous MOAP-1 interacts with exogenous SIRT2 and SIRT5. HEK293T cells were transiently transfected with FLAG-SIRT1-5 for 24 hours. Cells were harvested and cell lysates were immuno-precipitated with anti-MOAP-1 antibody. Cell lysates and immuno-precipitates were subjected to SDS-PAGE and analyzed with western blotting.

SIRT2 and SIRT 5 have previously been reported to be involved in the acetylation of metabolic enzymes in response to nutrient availability and hence have consequently emerged as integral components of the nutrient-sensing metabolic regulatory circuit (252). To determine whether the interaction between MOAP-1 and SIRT2/5 depends on nutrients availability, the protein-protein interaction profile of MOAP-1 and SIRT2/5 were investigated under serum starvation. As illustrated in Fig 5.4, interaction between endogenous MOAP-1 and SIRT5 was abolished under serum starvation while the interaction between MOAP-1 and SIRT2 remained unchanged. This may suggest that the MOAP-1:SIRT5 interaction is regulated by nutrient availability. In addition, consistent with previously reported data, MOAP-1 is up-regulated upon serum starvation and serum starvation-mediated up-

regulation of MOAP-1 is not affected by over-expression of SIRT2 and SIRT5 (Fig 5.4).

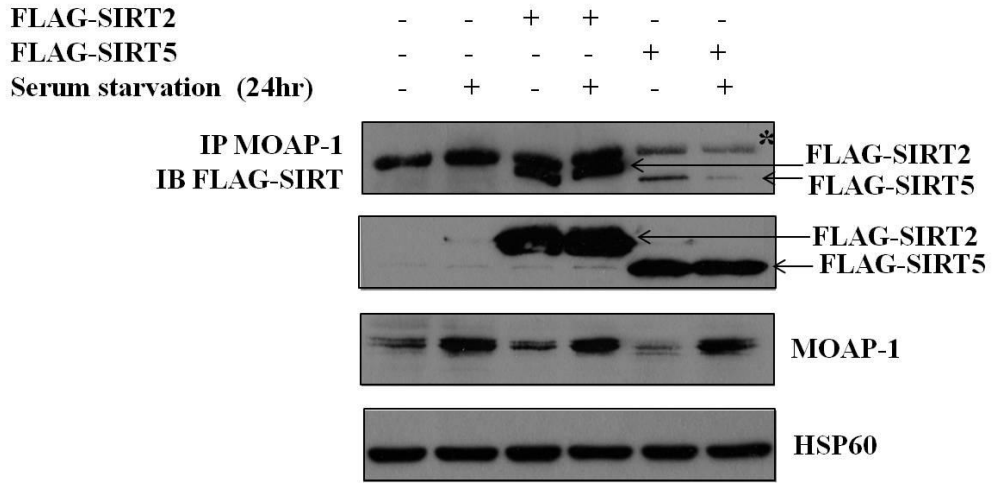

Figure 5.4 Serum starvation abolish the interaction between MOAP-1 and SIRT5. HEK293T cells were transiently transfected with FLAG-SIRT2 or FLAG-SIRT5 for 24 hours. Cells were then starved with serum free medium and harvested after 24 hours. Cells were harvested and cell lysates were immuno-precipitated with anti-MOAP-1 antibody. Cell lysates and immuno-precipitates were subjected to SDS-PAGE and analyzed with western blotting. HSP60 was used as a loading control to demonstrate that equal amounts of cell lysates was loaded into each lane. *denotes IGG bands.

In addition, endogenous interaction between SIRT2 and MOAP-1 was investigated in HEK293T cells while the endogenous interaction between MOAP-1 and SIRT5 remains to be determined in future work. Cells over-expressing SIRT2 were used as positive control to validate the specificity of the SIRT2 antibody. HEK293T cell lysates were immuno-precipitated with anti-MOAP-1 antibody and rabbit IgG antibody as negative control. Cell lysates and immuno-precipitates were subjected to SDS-PAGE and analyzed using western blotting. As demonstrated in Fig 5.5, endogenous interaction between MOAP-1 and SIRT2 was detected, suggesting that SIRT2 may indeed be a protein partner of MOAP-1.

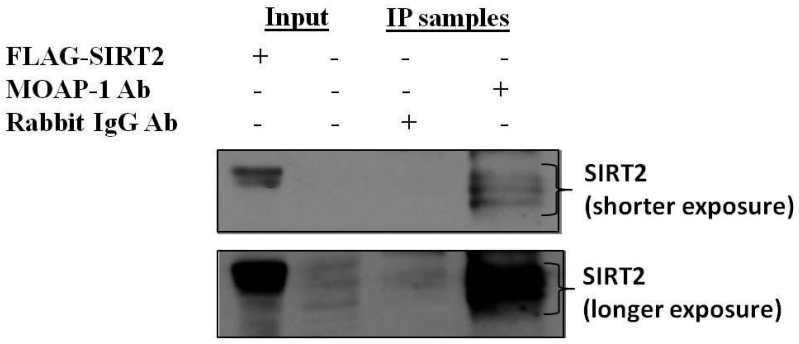

Figure 5.5 Endogenous interaction between MOAP-1 and SIRT2 was observed in HEK293T cells. HEK293T cell lysates were immuno-precipitated with anti-MOAP-1 antibody and rabbit IGG antibody as negative control. Cell lysates and immuno-precipitates were subjected to SDS-PAGE and analyzed with western blotting. Cells over-expressing SIRT2 were used as positive control to validate the specificity of the SIRT2 antibody.

5.2.3 SIRT5 suppresses ETOP-mediated up-regulation of MOAP-1

It has been well validated in our laboratory that proteasome inhibition and apoptotic stimuli can up-regulate endogenous protein level of MOAP-1. Since SIRT2 and SIRT5 are deacetylases and acetylation has been reported to compete with ubiquitination for lysine residues and hence regulate protein stability (253), we investigated whether over-expression of SIRT2 or SIRT5 affects the protein levels of MOAP-1 under DNA-damaging apoptotic stimulus, ETOP. Notably, over-expression of SIRT5, but not SIRT2, suppressed the up-regulation of MOAP-1 by ETOP (Fig 5.6) suggesting that SIRT5 may mediate the protein stability of MOAP-1 under DNA-damaging apoptotic stimuli.

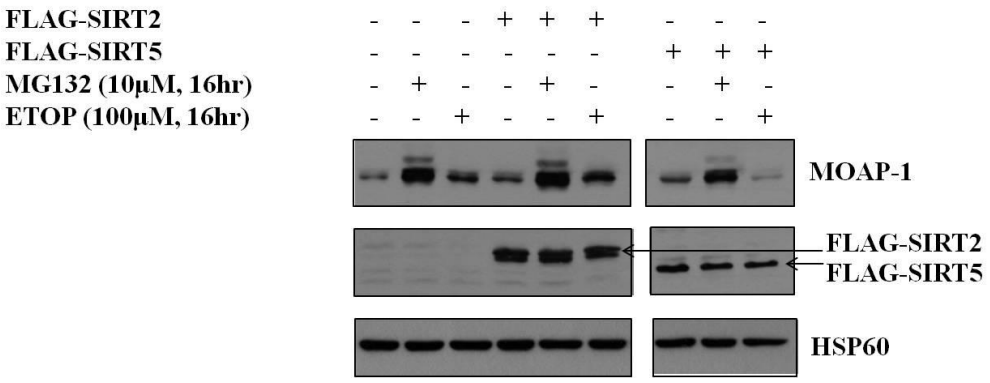

Figure 5.6 Over-expression of SIRT5 blocked MOAP-1 protein stabilization by ETOP. HEK293T cells were transiently transfected with FLAG-SIRT2 or FLAG-SIRT5 for 24 hours. Cells were then treated with 10 μM MG132 or 100 μM ETOP for 16 hours. Cells were harvested and protein samples were subjected to SDS-PAGE and analyzed with western blotting. HSP60 was used as a loading control to demonstrate that equal amounts of cell lysates was loaded into each lane.

5.3 Discussion

Sirtuins have emerged as a promising group of proteins which have been reported to play key roles in various cellular processes including, but not limited to, energy metabolism, apoptosis and cell proliferation. Since sirtuins use NAD^+ as a co-factor, they have been intimately linked to metabolic status of cells (254). In the same way, sirtuins have emerged as integral components of the nutrient-sensing metabolic regulatory circuit, mediating nutrient availability and the regulation of various metabolic enzymes (252).

Sub-cellular localization and protein-protein interaction profiling are both classical approaches used to study novel functions of a protein. In particular, protein-protein interactions are intrinsic to virtually all cellular processes and confer a large degree of regulation in the cells. As a result, roles and significance of a protein in the cell could be deduced by identifying its interaction network and subsequently the consequences of the interactions

(255). In an attempt to draft a framework to set directions for future investigations on elucidating novel functions of MOAP-1, we explored the sub-cellular localization and protein-protein interaction profiles of MOAP-1 and sirtuins in this study.

Confocal microscopy analysis revealed that SIRT2 and SIRT5 strongly co-localized with MOAP-1 in HeLa cells (Fig 5.1B&E and Fig 5.2) leading us to investigate for physical interaction between these proteins. Using co-immuno-precipitation assay, endogenous MOAP-1 was found to interact physically with exogenous SIRT2 and SIRT5 in HEK293T cells (Fig 5.3). Interestingly, serum starvation abolished the interaction between MOAP-1 and SIRT5 but not between MOAP-1 and SIRT2, suggesting that MOAP-1 may exist as a complex with SIRT2 under nutrient deprivation condition, mediating the regulation of various metabolic enzymes. Furthermore, endogenous interaction between MOAP-1 and SIRT2 was detected in HEK293T cells (Fig 5.5) lending further support to the notion that MOAP-1 could be participating with SIRT2 in many cellular functions.

Interestingly, SIRT5 selectively blocked ETOP-mediated up-regulation of MOAP-1 (Fig 5.6) but serum starvation-induced elevation of MOAP-1 was not affected by over-expression of SIRT5 (Fig. 5.4). This reinforces the notion that the underlying molecular steps and regulators involved in modulating the protein stability of MOAP-1 may be distinct for individual stimulus (67). In addition, studies have established that acetylation, similar to phosphorylation, can alter the protein conformation and hence manipulate whether a protein is recognized and targeted for degradation (256). Alternatively, acetylation may compete with ubiquitination for lysine residues and hence mediate the steady-

state turnover of a protein (253). Since SIRT5 possess deacetylation activity, it is conceivable that acetylation of MOAP-1 may be required for its ETOP-mediated up-regulation. Notably, it was reported that in addition to its function as a deacetylase, SIRT5 may also act as demalonylase and desuccinylase (153), giving rise possibility that MOAP-1 may be regulated by malonylation and succinylation too.

In addition, it was also reported that SIRT5 may have an anti-apoptotic role as knockdown of SIRT5 resulted in the down-regulation of Bcl-xL and a marked reduction in cell viability and an increase in apoptotic cell number (257). This study may lend some support to our finding as MOAP-1 has been shown to be an effector of Bax-mediated apoptosis. Hence, the suppression of ETOP-mediated up-regulation of MOAP-1 by SIRT5 may further reinforce its reported anti-apoptotic function.

Collectively, our data demonstrate SIRT2 and SIRT5 as novel interactors of MOAP-1 and suggest that in addition to ubiquitination and phosphorylation, MOAP-1 may be regulated by other PTMs such as acetylation, malonylation and succinylation.

CHAPTER 6

General discussion, conclusion and future work

6.1 GENERAL DISCUSSION

Protein abundance of MOAP-1 is regulated through the ubiquitin proteasome system (UPS) (67). Correspondingly, the up-regulation and ubiquitin-conjugated species of MOAP-1 can be readily observed in the presence of a proteasome inhibitor, MG132 (67). We showed the identification and characterization of tripartite motif protein 11 (TRIM11) as a putative E3 ubiquitin ligase of MOAP-1. TRIM11 physically associates with and degrades MOAP-1 by poly-ubiquitinating MOAP-1 for degradation through the UPS. Targeted inactivation of TRIM11 E3 ubiquitin ligase activity by alanine substitution of conserved cysteine and histidine residues in the RING domain abolished its ability to poly-ubiquitinate and degrade MOAP-1.

In the following chapter, we explored the prospective of phosphorylation as a regulatory mechanism for modulating the protein stability and function of MOAP-1. We showed that the protein stability of MOAP-1 is regulated by phosphorylation at serine-27 (Ser-27), Ser-29 and Ser-31. Constitutive phosphorylation at these sites by aspartate substitution prolongs the half-life of MOAP-1. In addition, we also identified the mitogen-activated protein kinase kinase (MAPKK also referred to as MEK1) as MOAP-1-associating partner that stabilizes MOAP-1 through extension of MOAP-1 half-life. Notably, constitutive active form of MEK1, MEK1Δ-mediated stabilization of MOAP-1 requires the constitutive phosphorylation at Ser-27, Ser-29 and Ser-31, supporting a role for MEK1 in modulating the protein stability of MOAP-1 through phosphorylation at these serine sites. Also, in agreement with its effect on enhancing MOAP-1 stability, over-

expression of MEK1Δ sensitizes HCT116 cells to TRAIL-induced Bax-activation and apoptosis. In this chapter, our data documented that phosphorylation is a regulatory mechanism in regulating the protein abundance and function of MOAP-1.

In the first two chapters of results, we have separately explored and examined the potential role of ubiquitination and phosphorylation in regulating protein stability and function of MOAP-1. It has been widely acknowledged that PTMs regulate protein function in a highly orchestrated cascade of ubiquitination/de-ubiquitination events that are intermingled with other PTMs such as phosphorylation/de-phosphorylation or acetylation/de-acetylation. Since phosphorylation of MOAP-1 has been shown to modulate its protein stability, it is highly conceivable that the phosphorylation status of MOAP-1 may regulate the interaction of MOAP-1 with its specific E3 ubiquitin ligase, thereby controlling its protein abundance (Fig 6.1).

Based on the experimental findings obtained in this study, we proposed that in healthy cells, TRIM11 associates with and degrades MOAP-1 by targeting MOAP-1 for degradation via the UPS. Since MOAP-1 has previously been demonstrated to be a critical effector for Bax-mediated apoptosis, elimination of MOAP-1 reduces the activation and oligomerization of Bax into the outer mitochondrial membrane thereby promoting cell survival. On contrary, DNA damaging agent, ETOP activates the MAPK signaling cascade leading to the sequential activation of MEK and ERK. Phosphorylation of MOAP-1 at Ser-27, Ser-29 and Ser31 stabilizes MOAP-1 and is required for MEK1-mediated stabilization of MOAP-1. However, the

kinase(s) specifically responsible for phosphorylating these sites remain to be determined. Up to date, the only known physiological substrates of MEK1/2 are ERK1/2 (258). Due to the substrate specificity of MEK1, it is unlikely that MEK1 itself functions as a MOAP-1 kinase. Instead, it is more likely that ERK1/2 may phosphorylate MOAP-1 directly or through activation of other downstream kinases. In contrary to our initial proposal that MOAP-1 may undergo a similar mode of regulation to Bim where phosphorylation is required for the subsequent ubiquitinated-mediated degradation, we proposed that phosphorylation of MOAP-1 at Ser-27, Ser-29 and Ser-31 inhibits the targeting of MOAP-1 by its E3 ubiquitin ligase for degradation through the UPS. The proposed disruption of interaction between MOAP-1 alanine mutant (S27,29,31A) and putative E3 ubiquitin ligase, TRIM11 remains to be validated in the future work. Correspondingly, under apoptotic condition, stabilized MOAP-1 associates with Bax and mediates its activation and oligomerization into the outer mitochondrial membrane which ultimately leads to mitochondrial outer membrane permeabilization, (MOMP), cytochrome c release and cell death.

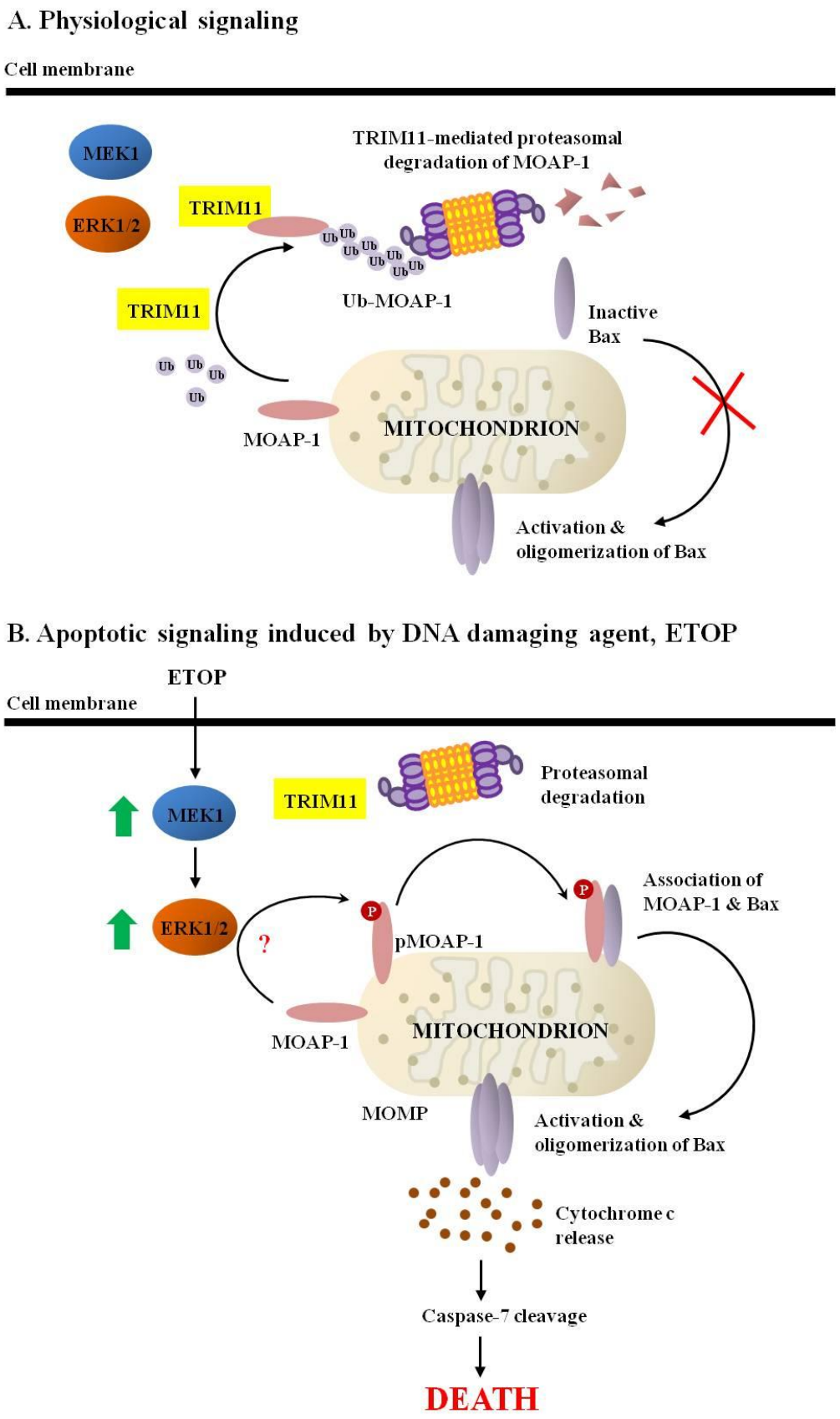

Figure 6.1 Proposed sequences of events during physiological condition (A) and ETOP-induced apoptosis (B). Figures are modified and adapted from (259).

In the last chapter of results, we identified SIRT2 and SIRT5, which are members of the sirtuin family of deacetylases, as novel interactors of MOAP-1. Our data also support a role for SIRT5 in mediating the regulation of MOAP-1 under DNA-damaging agent, ETOP. In addition, serum starvation abolished the interaction between MOAP-1 and SIRT5 suggesting a plausible role of MOAP-1 in modulating the activity of SIRT5 under normal physiological condition. The results obtained thus far are still insufficient to conclude whether acetylation may act as a regulatory mechanism to modulate the protein stability and function of MOAP-1.

In summary, the work presented in this thesis has identified TRIM11, MEK1 and SIRT5 as potential regulators of MOAP-1. Interestingly, the known functions of these three proteins are distinctly different. While SIRT5 has been reported to play a role in ammonia detoxification and exhibits deacetylase, demalonylase and desuccinylase activity, TRIM11 is believed to play a role in immunity and exhibits E3 ubiquitin ligase activity. In comparison, the role of MEK1 as a kinase has been demonstrated in regulating many fundamental cellular activities including cell survival, proliferation, differentiation and motility. The discrete distinction in the enzymatic activity of these potential regulators may open up windows of opportunities to strategize approaches to increase levels of MOAP-1 in cancer cells, thereby ensuring optimal Bax function in the mitochondrial-mediated apoptotic pathway.

6.2 FUTURE WORK

6.2.1 Identification and characterization of TRIM11 as a putative E3 ubiquitin ligase of MOAP-1

Knockdown of endogenous TRIM11 in HEK293T cells did not alter the protein abundance of MOAP-1. However, this may be due to the low endogenous expression of TRIM11 in HEK293T cells. More cell-lines should be screened to investigate the effect of TRIM11 knock-down on the protein abundance of MOAP-1 under resting and apoptotic condition.

In addition, MOAP-1 has been reported to be a substrate of multi-unit E3 ubiquitin ligase, APC/C^{Cdh1} (104). In the paper, the authors showed that knock-down of Cdh1, activator of APC/C, using siRNA significantly slowed down the degradation of ectopic MOAP-1. It remains to be investigated if double knockdown of both TRIM11 and APC/C^{Cdh1} will synergize or have a redundancy effect on the protein abundance and function of MOAP-1. Similarly, since the scope of this study covers only the post-translational regulation of MOAP-1, additive experiments will be needed to assess the possibility whether TRIM11 can affect the transcription and translation of MOAP-1. In addition, future study may also investigate the interplay between TRIM39 and TRIM11 in regulating the protein stability of MOAP-1 via the UPS. In a recent review, it was proposed that TRIM family of proteins may possess scaffolding capabilities and be involved in self- and hetero-interaction, giving rise to a complex TRIM biochemical activity (261). In a similar context, it was reported that TRIM21 interacts with TRIM5 to ubiquitinate and degrade TRIM5 in HEK293 cells (262). Hence, future work may investigate if

TRIM11 and TRIM39 possess similar E3 ligase-substrate relationship. In addition, since TRIM39 and TRIM11 have been shown to exert opposing effects of MOAP-1, it would be interesting to evaluate the protein abundance of MOAP-1 under co-expression of both TRIM proteins.

The field of de-ubiquitination is another area that may be looked into in the future work. At present, there is no evidence for a role of deubiquitinases in the molecular regulation of MOAP-1. Deubiquitinases catalyze the removal of ubiquitin moieties from proteins and hence may be used to nullify ubiquitylation signals, serving as an extra layer of control to regulate ubiquitination and to remove ubiquitin chains from substrate proteins prior to proteasomal degradation (263). It is possible that both ubiquitination and de-ubiquitination occur at a very rapid exchange process thereby providing an extremely dynamic ubiquitination response (263).

6.2.2 Evaluation of phosphorylation as a regulatory mechanism to modulate protein stability of MOAP-1

While the work presented in this thesis has investigated specifically on the potential role of phosphorylation in regulating the protein stability of MOAP-1, phosphorylation may also modulate many other aspects of a protein including its intrinsic functional activity, folding and conformation, oligomerization state, sub-cellular localization and its interaction with other cellular molecules in the cell. Future analysis may look into the impact of phosphorylation on the sub-cellular localization and apoptotic function of MOAP-1.

In the *in vitro* phosphorylation experiment, though a band corresponding to the molecular weight of GST-MOAP-1 was detected (Fig 4.2), mass spectrometry analysis of the brain lysates was not conducted to identify potential kinase(s) which may have phosphorylated MOAP-1 in the kinase reaction. This may be investigated in the future to identify downstream kinases of MEK which may function as direct kinase(s) of MOAP-1.

Constitutive active MEK1 associates with and stabilizes MOAP-1. In addition, phosphorylation of MOAP-1 at Ser-27, Ser-29 and Ser31 is required for MEK1-mediated stabilization of MOAP-1. However due to the substrate specificity of MEK1, it is unlikely that MEK1 itself functions as a MOAP-1 kinase. Therefore, future work can be carried out to identify and validate if ERK1/2 or its downstream kinase are putative kinase of MOAP-1. In addition, knock-down gene analysis of MEK could be conducted to further support and validate the role of MEK in regulating the protein stability of MOAP-1. In addition, constitutive phosphorylation of MOAP-1 at Ser-27, Ser-29 and Ser31 by aspartate substitution stabilizes MOAP-1. Since our data showed that TRIM11 interacts with and degrades MOAP-1 through the UPS it is proposed that future work can be carried out to investigate if phosphorylation at the three serine sites abolishes the interaction between MOAP-1 and TRIM11, thereby preventing the poly-ubiquitination and consequent degradation of MOAP-1.

Similar to de-ubiquitination, the field of de-phosphorylation is an area that can be further examined. Up to date, there is no evidence to support a role of phosphatase in the molecular regulation of MOAP-1. Similar to many other

Bcl-2 family proteins which have been reported to be regulated by reversible phosphorylation, it is conceivable that both phosphorylation and de-phosphorylation work cooperatively on MOAP-1 to regulate its protein abundance and function as a pro-apoptotic protein in different cellular contexts. Correspondingly, since the scope of this study covers only the post-translational regulation of MOAP-1, additive experiments will be needed to assess the possibility whether MEK1 can affect the transcription and translation of MOAP-1.

6.2.3 Identification of Sirtuins as novel interactors and regulators of MOAP-1

SIRT2 and SIRT5 are members of the sirtuin family of deacetylases. Novel interactions between endogenous MOAP-1 and SIRT2 and SIRT5 were observed. Given that SIRT2 and SIRT5 have been reported to deacetylate many key metabolic proteins, it remains to be investigated whether MOAP-1 may be a substrate of deacetylation by either or both SIRT2 and SIRT5. Notably, SIRT2 has been linked to the regulation of many cellular processes such as adipocyte differentiation through the modulation of FOXO1 acetylation and activity (264). It would be interesting to investigate if MOAP-1 may synergize with SIRT2, promoting its regulation on FOXO1 thereby playing a role in controlling adipose tissue mass and function.

In addition, it is an interesting observation that serum starvation abolished the interaction between endogenous MOAP-1 and SIRT5. Notably, SIRT5 has been reported to deacetylate and activate carbamoyl phosphate synthetase 1 (CPS1) after 48 hours of fasting (172). CPS1 catalyzes the initial

step of the urea cycle for ammonia detoxification and disposal and hence is a committed and regulated enzyme of the urea cycle (265,266). It would be interesting to investigate if MOAP-1 associates with SIRT5 to sequester SIRT5 under high nutrients condition. Whereas during fasting condition, the interaction between MOAP-1 and SIRT5 is promptly disrupted, releasing SIRT5 to deacetylate and activate CPS1. Interestingly, SIRT5 selectively blocked ETOP-mediated up-regulation of MOAP-1. Since SIRT5 possess deacetylation activity, it is conceivable that acetylation of MOAP-1 may be required for its ETOP-mediated up-regulation. Notably, it was reported that in addition to its function as a deacetylase, SIRT5 may also act as demalonylase and desuccinylase (153), giving rise possibility that MOAP-1 may be regulated by malonylation and succinylation too. These are areas that could be investigated in the future work.

6.3 CONCLUSION

The findings of this Ph.D. study have provided further insights to enhance our understanding of the mechanisms that serve to regulate MOAP-1 stability (Fig 6.1). While TRIM11 may function as an E3 ubiquitin ligase to target MOAP-1 to the UPS, the activation of MEK1 potentially results in the phosphorylation of MOAP-1 which prevents its degradation via the UPS. Lastly, more work will be required to verify if direct deacetylation of MOAP-1 by SIRT5 can be observed. The identification of these novel molecules including TRIM11, MEK1 and SIRT5 may serve as valuable leads to reveal the detailed mechanisms by which apoptotic signals regulate protein stability of MOAP-1. As such, this would aid in strategizing approaches to elevate levels of MOAP-1 in cancer cells, thereby ensuring optimal Bax functioning and consequently increasing the sensitivity of cancer cells to chemo-therapeutic treatments.

BIBLIOGRAPHY

1. Jesenberger, V., and Jentsch, S. (2002) Deadly encounter: Ubiquitin meets apoptosis. *Nature Reviews Molecular Cell Biology* **3**, 112-121
2. Peter, M. E., Heufelder, A. E., and Hengartner, M. O. (1997) Advances in apoptosis research. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 12736-12737
3. Metzstein, M. M., Stanfield, G. M., and Horvitz, H. R. (1998) Genetics of programmed cell death in C. elegans: past, present and future. *Trends Genet* **14**, 410-416
4. Vaux, D. L., and Strasser, A. (1996) The molecular biology of apoptosis. *Proc. Natl Acad. Sci. USA* **93**, 2239-2244
5. Vaux, D. L., and Korsmeyer, S. J. (1999) Cell death in development. *Cell* **96**, 245-254
6. Strasser, A., O'Connor, L., and Dixit, V. M. (2000) Apoptosis signaling. *Annu. Rev. Biochem.* **69**, 217-245
7. Hacker, G. (2000) The morphology of apoptosis. *Cell and Tissue Research* **301**, 5-17
8. Kroemer, G., El-Deiry, W. S., Golstein, P., Peter, M. E., Vaux, D., Vandenabeele, P., Zhivotovsky, B., Blagosklonny, M. V., Malorni, W., Knight, R. A., Piacentini, M., Nagata, S., Melino, G., and Death, N. C. o. C. (2005) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. *Cell Death Differ* **12 Suppl 2**, 1463-1467
9. Taatjes, D. J., Sobel, B. E., and Budd, R. C. (2008) Morphological and cytochemical determination of cell death by apoptosis. *Histochemistry and Cell Biology* **129**, 33-43
10. D'Emilio, A., Biagiotti, L., Burattini, S., Battistelli, M., Canonico, B., Evangelisti, C., Ferri, P., Papa, S., Martelli, A. M., and Falcieri, E. (2010) Morphological and biochemical patterns in skeletal muscle apoptosis. *Histology and Histopathology* **25**, 21-32
11. Mates, J. M., Segura, J. A., Alonso, F. J., and Marquez, J. (2012) Oxidative stress in apoptosis and cancer: an update. *Archives of Toxicology* **86**, 1649-1665
12. Hotchkiss, R. S., Strasser, A., McDunn, J. E., and Swanson, P. E. (2009) Cell Death. *New England Journal of Medicine* **361**, 1570-1583
13. Circu, M. L., and Aw, T. Y. (2012) Glutathione and modulation of cell apoptosis. *Biochimica Et Biophysica Acta-Molecular Cell Research* **1823**, 1767-1777
14. Taylor, R. C., Cullen, S. P., and Martin, S. J. (2008) Apoptosis: controlled demolition at the cellular level. *Nature Reviews Molecular Cell Biology* **9**, 231-241
15. Horvitz, H. R. (1999) Genetic control of programmed cell death in the nematode Caenorhabditis elegans. *Cancer Research* **59**, 1701S-1706S
16. Cory, S., and Adams, J. M. (2002) The Bcl2 family: regulators of the cellular life-or-death switch. *Nature Rev. Cancer* **2**, 647-656
17. Thompson, C. B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* **267**, 1456-1462
18. Fischer, U., and Schulze-Osthoff, K. (2005) New approaches and therapeutics targeting apoptosis in disease. *Pharmacological Reviews* **57**, 187-215
19. Nijhawan, D., Honarpour, N., and Wang, X. D. (2000) Apoptosis in neural development and disease. *Annual Review of Neuroscience* **23**, 73-87

20. Vila, M., and Przedborski, S. (2003) Targeting programmed cell death in neurodegenerative diseases. *Nature Reviews Neuroscience* **4**, 365-375
21. Thompson, C. B. (1995) APOPTOSIS IN THE PATHOGENESIS AND TREATMENT OF DISEASE. *Science* **267**, 1456-1462
22. Reed, J. C. (1994) BCL-2 AND THE REGULATION OF PROGRAMMED CELL-DEATH. *Journal of Cell Biology* **124**, 1-6
23. Kroemer, G., Galluzzi, L., and Brenner, C. (2007) Mitochondrial membrane permeabilization in cell death. *Physiol Rev* **87**, 99-163
24. Neutzner, A., Li, S. N., Xu, S., and Karbowski, M. (2012) The ubiquitin/proteasome system-dependent control of mitochondrial steps in apoptosis. *Seminars in Cell & Developmental Biology* **23**, 499-508
25. Green, D. R., and Kroemer, G. (2004) The pathophysiology of mitochondrial cell death. *Science* **305**, 626-629
26. Crow, M. T., Mani, K., Nam, Y. J., and Kitsis, R. N. (2004) The mitochondrial death pathway and cardiac myocyte apoptosis. *Circ Res* **95**, 957-970
27. Yin, X. M. (1999) Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature* **400**, 886-891
28. Kaufmann, T., Tai, L., Ekert, P. G., Huang, D. C. S., Norris, F., Lindemann, R. K., Johnstone, R. W., Dixit, V. M., and Strasser, A. (2007) The BH3-Only Protein Bid Is Dispensable for DNA Damage- and Replicative Stress-Induced Apoptosis or Cell-Cycle Arrest. *Cell* **129**, 423-433
29. Fulda, S., Galluzzi, L., and Kroemer, G. (2010) Targeting mitochondria for cancer therapy. *Nature Reviews Drug Discovery* **9**, 447-464
30. Martinou, J. C., and Youle, R. J. (2011) Mitochondria in Apoptosis: Bcl-2 Family Members and Mitochondrial Dynamics. *Developmental Cell* **21**, 92-101
31. Gogvadze, V., Orrenius, S., and Zhivotovsky, B. (2008) Mitochondria in cancer cells: what is so special about them? *Trends in Cell Biology* **18**, 165-173
32. Taylor, R. W., and Turnbull, D. M. (2005) Mitochondrial DNA mutations in human disease. *Nature Reviews Genetics* **6**, 389-402
33. Galluzzi, L., Morselli, E., Kepp, O., Vitale, I., Rigoni, A., Vacchelli, E., Michaud, M., Zischka, H., Castedo, M., and Kroemer, G. (2010) Mitochondrial gateways to cancer. *Molecular Aspects of Medicine* **31**, 1-20
34. Blanco, F. J., Rego, I., and Ruiz-Romero, C. (2011) The role of mitochondria in osteoarthritis. *Nature Reviews Rheumatology* **7**, 161-169
35. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) APOPTOSIS - BASIC BIOLOGICAL PHENOMENON WITH WIDE-RANGING IMPLICATIONS IN TISSUE KINETICS. *British Journal of Cancer* **26**, 239-&
36. Jacobson, M. D., Burne, J. F., King, M. P., Miyashita, T., Reed, J. C., and Raff, M. C. (1993) BCL-2 BLOCKS APOPTOSIS IN CELLS LACKING MITOCHONDRIAL-DNA. *Nature* **361**, 365-369
37. Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D., and Korsmeyer, S. J. (1990) BCL-2 IS AN INNER MITOCHONDRIAL-MEMBRANE PROTEIN THAT BLOCKS PROGRAMMED CELL-DEATH. *Nature* **348**, 334-336
38. Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Milliman, C. L., and Korsmeyer, S. J. (1993) BCL-2 FUNCTIONS IN AN ANTIOXIDANT PATHWAY TO PREVENT APOPTOSIS. *Cell* **75**, 241-251
39. Newmeyer, D. D., Farschon, D. M., and Reed, J. C. (1994) CELL-FREE APOPTOSIS IN XENOPUS EGG EXTRACTS - INHIBITION BY BCL-2 AND

REQUIREMENT FOR AN ORGANELLE FRACTION ENRICHED IN MITOCHONDRIA. *Cell* **79**, 353-364
40. Kroemer, G., Petit, P., Zamzami, N., Vayssiere, J. L., and Mignotte, B. (1995) THE BIOCHEMISTRY OF PROGRAMMED CELL-DEATH. *Faseb Journal* **9**, 1277-1287
41. Zamzami, N., Susin, S. A., Marchetti, P., Hirsch, T., GomezMonterrey, I., Castedo, M., and Kroemer, G. (1996) Mitochondrial control of nuclear apoptosis. *Journal of Experimental Medicine* **183**, 1533-1544
42. Liu, X. S., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. D. (1996) Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome c. *Cell* **86**, 147-157
43. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. D. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479-489
44. Adams, J. M., and Cory, S. (2007) The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* **26**, 1324-1337
45. Chan, S. L., and Yu, V. C. (2004) Proteins of the Bcl-2 family in apoptosis signalling: From mechanistic insights to therapeutic opportunities. *Clinical and Experimental Pharmacology and Physiology* **31**, 119-128
46. Bakhshi, A., Jensen, J. P., Goldman, P., Wright, J. J., McBride, O. W., Epstein, A. L., and Korsmeyer, S. J. (1985) Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell* **41**, 899-906
47. Boise, L. H., González-García, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nuñez, G., and Thompson, C. B. (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74**, 597-608
48. Willis, S. N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J. I., Adams, J. M., and Huang, D. C. S. (2005) Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* **19**, 1294-1305
49. Willis, S. N., Fletcher, J. I., Kaufmann, T., van Delft, M. F., Chen, L., Czabotar, P. E., Ierino, H., Lee, E. F., Fairlie, W. D., Bouillet, P., Strasser, A., Kluck, R. M., Adams, J. M., and Huang, D. C. S. (2007) Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science* **315**, 856-859
50. Letai, A. (2002) Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* **2**, 183-192
51. Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**, 609-619
52. Certo, M., Moore, V. D., Nishino, M., Wei, G., Korsmeyer, S., Armstrong, S. A., and Letai, A. (2006) Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* **9**, 351-365
53. Kim, H., Rafiuddin-Shah, M., Tu, H. C., Jeffers, J. R., Zambetti, G. P., Hsieh, J. J. D., and Cheng, E. H. Y. (2006) Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nature Cell Biology* **8**, 1348-U1319

54. Ren, D. C., Tu, H. C., Kim, H., Wang, G. X., Bean, G. R., Takeuchi, O., Jeffers, J. R., Zambetti, G. P., Hsieh, J. J. D., and Cheng, E. H. Y. (2010) BID, BIM, and PUMA Are Essential for Activation of the BAX- and BAK-Dependent Cell Death Program. *Science* **330**, 1390-1393
55. Hsu, Y. T., Wolter, K. G., and Youle, R. J. (1997) Cytosol-to-membrane redistribution of Bax and Bcl-X-L during apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 3668-3672
56. Wolter, K. G. (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. *J. Cell Biol.* **139**, 1281-1292
57. Wang, X. (2001) The expanding role of mitochondria in apoptosis. *Genes Dev* **15**, 2922-2933
58. Chipuk, J. E., and Green, D. R. (2008) How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol* **18**, 157-164
59. Danial, N. N., and Korsmeyer, S. J. (2004) Cell death: Critical control points. *Cell* **116**, 205-219
60. Strasser, A. (2005) The role of BH3-only proteins in the immune system. *Nat Rev Immunol* **5**, 189-200
61. Willis, S. N., and Adams, J. M. (2005) Life in the balance: how BH3-only proteins induce apoptosis. *Current Opinion in Cell Biology* **17**, 617-625
62. Wei, M. C., Zong, W. X., Cheng, E. H. Y., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacCregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001) Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. *Science* **292**, 727-730
63. LeBlanc, H., Lawrence, D., Varfolomeev, E., Totpal, K., Morlan, J., Schow, P., Fong, S., Schwall, R., Sinicropi, D., and Ashkenazi, A. (2002) Tumor-cell resistance to death receptor-induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. *Nature Medicine* **8**, 274-281
64. Zhang, L., Yu, J., Park, B. H., Kinzler, K. W., and Vogelstein, B. (2000) Role of BAX in the apoptotic response to anticancer agents. *Science* **290**, 989-+
65. Tan, K. O., Tan, K. M. L., Chan, S. L., Yee, K. S. Y., Bevort, M., Ang, K. C., and Yu, V. C. (2001) MAP-1, a novel proapoptotic protein containing a BH3-like motif that associates with Bax through its Bcl-2 homology domains. *Journal of Biological Chemistry* **276**, 2802-2807
66. Tan, K. O., Fu, N. Y., Sukumaran, S. K., Chan, S. L., Kang, J. H., Poon, K. L., Chen, B. S., and Yu, V. C. (2005) MAP-1 is a mitochondrial effector of Bax. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 14623-14628
67. Fu, N. Y., Sukumaran, S. K., and Yu, V. C. (2007) Inhibition of ubiquitin-mediated degradation of MOAP-1 by apoptotic stimuli promotes Bax function in mitochondria. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 10051-10056
68. Baksh, S., Tommasi, S., Fenton, S., Yu, V. C., Martins, L. M., Pfeifer, G. P., Latif, F., Downward, J., and Neel, B. G. (2005) The tumor suppressor RASSF1A and MAP-1 link death receptor signaling to bax conformational change and cell death. *Molecular Cell* **18**, 637-650
69. Kutuk, O., and Letai, A. (2008) Regulation of Bcl-2 family proteins by posttranslational modifications. *Current Molecular Medicine* **8**, 102-118
70. Hunter, T. (1995) PROTEIN-KINASES AND PHOSPHATASES - THE YIN AND YANG OF PROTEIN-PHOSPHORYLATION AND SIGNALING. *Cell* **80**, 225-236

71. Walsh, G., and Jefferis, R. (2006) Post-translational modifications in the context of therapeutic proteins. *Nature Biotechnology* **24**, 1241-1252
72. Nijhawan, D., Fang, M., Traer, E., Zhong, Q., Gao, W. H., Du, F. H., and Wang, X. D. (2003) Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. *Genes & Development* **17**, 1475-1486
73. Ding, Q. Q., He, X. G., Hsu, J. M., Xia, W. Y., Chen, C. T., Li, L. Y., Lee, D. F., Liu, J. C., Zhong, Q., Wang, X. D., and Hung, M. C. (2007) Degradation of Mcl-1 by beta-TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization. *Molecular and Cellular Biology* **27**, 4006-4017
74. Schwickart, M., Huang, X. D., Lill, J. R., Liu, J. F., Ferrando, R., French, D. M., Maecker, H., O'Rourke, K., Bazan, F., Eastham-Anderson, J., Yue, P., Dornan, D., Huang, D. C. S., and Dixit, V. M. (2010) Deubiquitinase USP9X stabilizes MCL1 and promotes tumour cell survival. *Nature* **463**, 103-U114
75. Pickart, C. M. (2001) Mechanisms underlying ubiquitination. *Annual Review of Biochemistry* **70**, 503-533
76. Komander, D., and Rape, M. (2012) The Ubiquitin Code. *Annual Review of Biochemistry, Vol 81* **81**, 203-229
77. Haglund, K., and Dikic, I. (2005) Ubiquitylation and cell signaling. *Embo Journal* **24**, 3353-3359
78. Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dikic, I. (2003) Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nature Cell Biology* **5**, 461-466
79. Ciechanover, A. (1998) The ubiquitin-proteasome pathway: on protein death and cell life. *Embo Journal* **17**, 7151-7160
80. Shen, M., Schmitt, S., Buac, D., and Dou, Q. P. (2013) Targeting the ubiquitin-proteasome system for cancer therapy. *Expert Opinion on Therapeutic Targets* **17**, 1091-1108
81. Lee, D. H., and Goldberg, A. L. (1998) Proteasome inhibitors: valuable new tools for cell biologists. *Trends in Cell Biology* **8**, 397-403
82. Kirisako, T., Kamei, K., Murata, S., Kato, M., Fukumoto, H., Kanie, M., Sano, S., Tokunaga, F., Tanaka, K., and Iwai, K. (2006) A ubiquitin ligase complex assembles linear polyubiquitin chains. *Embo Journal* **25**, 4877-4887
83. Clague, M. J., and Urbe, S. (2010) Ubiquitin: Same Molecule, Different Degradation Pathways. *Cell* **143**, 682-685
84. Hershko, A., and Ciechanover, A. (1992) THE UBIQUITIN SYSTEM FOR PROTEIN-DEGRADATION. *Annual Review of Biochemistry* **61**, 761-807
85. Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000) Recognition of the polyubiquitin proteolytic signal. *Embo Journal* **19**, 94-102
86. Behrends, C., and Harper, J. W. (2011) Constructing and decoding unconventional ubiquitin chains. *Nature Structural & Molecular Biology* **18**, 520-528
87. Dammer, E. B., Na, C. H., Xu, P., Seyfried, N. T., Duong, D. M., Cheng, D. M., Gearing, M., Rees, H., Lah, J. J., Levey, A. I., Rush, J., and Peng, J. M. (2011) Polyubiquitin Linkage Profiles in Three Models of Proteolytic Stress Suggest the Etiology of Alzheimer Disease. *Journal of Biological Chemistry* **286**, 10457-10465
88. Ikeda, F., and Dikic, I. (2008) Atypical ubiquitin chains: new molecular signals - 'Protein modifications: Beyond the usual suspects' review series. *Embo Reports* **9**, 536-542
89. Randow, F., and Lehner, P. J. (2009) Viral avoidance and exploitation of the ubiquitin system. *Nature Cell Biology* **11**, 527-U535

90. Koga, H., Kaushik, S., and Cuervo, A. M. (2011) Protein homeostasis and aging: The importance of exquisite quality control. *Ageing Research Reviews* **10**, 205-215
91. Hochstrasser, M. (2009) Origin and function of ubiquitin-like proteins. *Nature* **458**, 422-429
92. Li, W., Bengtson, M. H., Ulbrich, A., Matsuda, A., Reddy, V. A., Orth, A., Chanda, S. K., Batalov, S., and Joazeiro, C. A. P. (2008) Genome-Wide and Functional Annotation of Human E3 Ubiquitin Ligases Identifies MULAN, a Mitochondrial E3 that Regulates the Organelle's Dynamics and Signaling. *Plos One* **3**
93. Frescas, D., and Pagano, M. (2008) Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. *Nature Reviews Cancer* **8**, 438-449
94. Lipkowitz, S., and Weissman, A. M. (2011) RINGs of good and evil: RING finger ubiquitin ligases at the crossroads of tumour suppression and oncogenesis. *Nature Reviews Cancer* **11**, 629-643
95. Bernassola, F., Karin, M., Ciechanover, A., and Melino, G. (2008) The HECT family of E3 ubiquitin ligases: Multiple players in cancer development. *Cancer Cell* **14**, 10-21
96. Huibregtse, J. M., Scheffner, M., Beaudenon, S., and Howley, P. M. (1995) A FAMILY OF PROTEINS STRUCTURALLY AND FUNCTIONALLY RELATED TO THE E6-AP UBIQUITIN PROTEIN LIGASE. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 2563-2567
97. Rotin, D., and Kumar, S. (2009) Physiological functions of the HECT family of ubiquitin ligases. *Nature Reviews Molecular Cell Biology* **10**, 398-409
98. Borden, K. L. B., Boddy, M. N., Lally, J., Oreilly, N. J., Martin, S., Howe, K., Solomon, E., and Freemont, P. S. (1995) THE SOLUTION STRUCTURE OF THE RING FINGER DOMAIN FROM THE ACUTE PROMYELOCYTIC LEUKEMIA PROTO-ONCOPROTEIN PML. *Embo Journal* **14**, 1532-1541
99. Barlow, P. N., Luisi, B., Milner, A., Elliott, M., and Everett, R. (1994) STRUCTURE OF THE C3HC4 DOMAIN BY H-1-NUCLEAR MAGNETIC-RESONANCE SPECTROSCOPY - A NEW STRUCTURAL CLASS OF ZINC-FINGER. *Journal of Molecular Biology* **237**, 201-211
100. Reymond A Fau - Meroni, G., Meroni G Fau - Fantozzi, A., Fantozzi A Fau - Merla, G., Merla G Fau - Cairo, S., Cairo S Fau - Luzi, L., Luzi L Fau - Riganelli, D., Riganelli D Fau - Zanaria, E., Zanaria E Fau - Messali, S., Messali S Fau - Cainarca, S., Cainarca S Fau - Guffanti, A., Guffanti A Fau - Minucci, S., Minucci S Fau - Pelicci, P. G., Pelicci Pg Fau - Ballabio, A., and Ballabio, A. The tripartite motif family identifies cell compartments.
101. Thompson, S., Pearson, A. N., Ashley, M. D., Jessick, V., Murphy, B. M., Gafken, P., Henshall, D. C., Morris, K. T., Simon, R. P., and Meller, R. (2011) Identification of a Novel Bcl-2-interacting Mediator of Cell Death (Bim) E3 Ligase, Tripartite Motif-containing Protein 2 (TRIM2), and Its Role in Rapid Ischemic Tolerance-induced Neuroprotection. *Journal of Biological Chemistry* **286**, 19331-19339
102. Ley, R., Balmanno, K., Hadfield, K., Weston, C., and Cook, S. J. (2003) Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. *J. Biol. Chem.* **278**, 18811-18816
103. Lee, S. S., Fu, N. Y., Sukumaran, S. K., Wan, K. F., Wan, Q., and Yu, V. C. (2009) TRIM39 is a MOAP-1-binding protein that stabilizes MOAP-1 through

inhibition of its poly-ubiquitination process. *Experimental Cell Research* **315**, 1313-1325
104. Huang, N. J., Zhang, L. G., Tang, W. L., Chen, C., Yang, C. S., and Kornbluth, S. (2012) The Trim39 ubiquitin ligase inhibits APC/C-Cdh1-mediated degradation of the Bax activator MOAP-1. *Journal of Cell Biology* **197**, 361-367
105. Ubersax, J. A., and Ferrell, J. E. (2007) Mechanisms of specificity in protein phosphorylation. *Nature Reviews Molecular Cell Biology* **8**, 530-541
106. Bode, A. M., and Dong, Z. G. (2004) Post-translational modification of p53 in tumorigenesis. *Nature Reviews Cancer* **4**, 793-805
107. Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) The protein kinase complement of the human genome. *Science* **298**, 1912-+
108. Niemi, N. M., and MacKeigan, J. P. (2013) Mitochondrial Phosphorylation in Apoptosis: Flipping the Death Switch. *Antioxidants & Redox Signaling* **19**, 572-582
109. Cohen, P., and Tcherpakov, M. (2010) Will the Ubiquitin System Furnish as Many Drug Targets as Protein Kinases? *Cell* **143**, 686-693
110. Mustelin, T., Vang, T., and Bottini, N. (2005) Protein tyrosine phosphatases and the immune response. *Nature Reviews Immunology* **5**, 43-57
111. Shaul, Y. D., and Seger, R. (2007) The MEK/ERK cascade: From signaling specificity to diverse functions. *Biochimica Et Biophysica Acta-Molecular Cell Research* **1773**, 1213-1226
112. Seger, R., and Krebs, E. G. (1995) PROTEIN KINASES .7. THE MAPK SIGNALING CASCADE. *Faseb Journal* **9**, 726-735
113. Raman, M., and Cobb, M. H. (2003) MAP kinase modules: Many roads home. *Current Biology* **13**, R886-R888
114. Santarpia, L., Lippman, S. M., and El-Naggar, A. K. (2012) Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy. *Expert Opinion on Therapeutic Targets* **16**, 103-119
115. McCubrey, J. A., Steelman, L. S., Chappell, W. H., Abrams, S. L., Wong, E. W. T., Chang, F., Lehmann, B., Terrian, D. M., Milella, M., Tafuri, A., Stivala, F., Libra, M., Basecke, J., Evangelisti, C., Martelli, A. M., and Franklin, R. A. (2007) Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochimica Et Biophysica Acta-Molecular Cell Research* **1773**, 1263-1284
116. Shaw, R. J., and Cantley, L. C. (2006) Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature* **441**, 424-430
117. Wellbrock, C., Karasarides, M., and Marais, R. (2004) The RAF proteins take centre stage. *Nature Reviews Molecular Cell Biology* **5**, 875-885
118. Chong, H., Vikis, H. G., and Guan, K. L. (2003) Mechanisms of regulating the Raf kinase family. *Cellular Signalling* **15**, 463-469
119. Crews, C. M., Alessandrini, A., and Erikson, R. L. (1992) THE PRIMARY STRUCTURE OF MEK, A PROTEIN-KINASE THAT PHOSPHORYLATES THE ERK GENE-PRODUCT. *Science* **258**, 478-480
120. Meloche, S., and Pouyssegur, J. (2007) The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. *Oncogene* **26**, 3227-3239
121. Mebratu, Y., and Tesfaigzi, Y. (2009) How ERK1/2 activation controls cell proliferation and cell death is subcellular localization the answer? *Cell Cycle* **8**, 1168-1175

122. Kim, H. J., and Bar-Sagi, D. (2004) Modulation of signalling by sprouty: A developing story. *Nature Reviews Molecular Cell Biology* **5**, 441-450
123. Luo, J., Manning, B. D., and Cantley, L. C. (2003) Targeting the PI3K-Akt pathway in human cancer: rationale and promise. *Cancer Cell* **4**, 257-262
124. Zhao, L., and Vogt, P. K. (2008) Class I PI3K in oncogenic cellular transformation. *Oncogene* **27**, 5486-5496
125. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* **278**, 687-689
126. Datta, S. R. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**, 231-241
127. Zha, J. P., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BGL-X(L). *Cell* **87**, 619-628
128. Maurer, U., Charvet, C., Wagman, A. S., Dejardin, E., and Green, D. R. (2006) Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Molecular Cell* **21**, 749-760
129. Deng, X. M., Ruvolo, P., Carr, B., and May, W. S. (2000) Survival function of ERK1/2 as IL-3-activated, staurosporine-resistant Bcl2 kinases. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 1578-1583
130. McCubrey, J. A., Steelman, L. S., Abrams, S. L., Lee, J. T., Chang, F. M., Bertrand, F. E., Navolanic, P. M., Terrian, D. M., Franklin, R. A., D'Assoro, A. B., Salisbury, J. L., Mazzarino, M. C., Stivala, F., and Libra, M. (2006) Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. in *Advances in Enzyme Regulation, Vol 46, Proceedings* (Weber, G., Weber, C. E. F., and Cocco, L. eds.). pp 249-279
131. Britten, C. D. (2013) PI3K and MEK inhibitor combinations: examining the evidence in selected tumor types. *Cancer Chemotherapy and Pharmacology* **71**, 1395-1409
132. Engelman, J. A. (2009) Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nature Reviews Cancer* **9**, 550-562
133. Vidall, G., Gershey, E. L., and Allfrey, V. G. (1968) CHEMICAL STUDIES OF HISTONE ACETYLATION - DISTRIBUTION OF EPSILON-N-ACETYLLYSINE IN CALF THYMUS HISTONES. *Journal of Biological Chemistry* **243**, 6361-&
134. Kleff, S., Andrulis, E. D., Anderson, C. W., and Sternglanz, R. (1995) IDENTIFICATION OF A GENE ENCODING A YEAST HISTONE H4 ACETYLTRANSFERASE. *Journal of Biological Chemistry* **270**, 24674-24677
135. Peserico, A., and Simone, C. (2011) Physical and Functional HAT/HDAC Interplay Regulates Protein Acetylation Balance. *Journal of Biomedicine and Biotechnology*
136. Arnesen, T., Van Damme, P., Polevoda, B., Helsens, K., Evjenth, R., Colaert, N., Varhaug, J. E., Vandekerckhove, J., Lillehaug, J. R., Sherman, F., and Gevaert, K. (2009) Proteomics analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 8157-8162
137. Kouzarides, T. (2000) Acetylation: a regulatory modification to rival phosphorylation? *Embo Journal* **19**, 1176-1179

138. Weinert, B. T., Wagner, S. A., Horn, H., Henriksen, P., Liu, W. S. R., Olsen, J. V., Jensen, L. J., and Choudhary, C. (2011) Proteome-Wide Mapping of the Drosophila Acetylome Demonstrates a High Degree of Conservation of Lysine Acetylation. *Science Signaling* **4**
139. Seto, E., Yuan, M., Zhang, M., Glozak, M., Sengupta, N., Villagra, A., Wong, P., Ferraro, B., and Rezai-Zadeh, N. (2006) Acetylation and deacetylation of non-histone proteins. *Faseb Journal* **20**, A1473-A1473
140. Spange, S., Wagner, T., Heinzel, T., and Kramer, O. H. (2009) Acetylation of non-histone proteins modulates cellular signalling at multiple levels. *International Journal of Biochemistry & Cell Biology* **41**, 185-198
141. Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V., and Mann, M. (2009) Lysine Acetylation Targets Protein Complexes and Co-Regulates Major Cellular Functions. *Science* **325**, 834-840
142. Kim, S. C., Sprung, R., Chen, Y., Xu, Y. D., Ball, H., Pei, J. M., Cheng, T. L., Kho, Y., Xiao, H., Xiao, L., Grishin, N. V., White, M., Yang, X. J., and Zhao, Y. M. (2006) Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. *Molecular Cell* **23**, 607-618
143. Caron, C., Boyault, C., and Khochbin, S. (2005) Regulatory cross-talk between lysine acetylation and ubiquitination: role in the control of protein stability. *Bioessays* **27**, 408-415
144. Gregoretti, I. V., Lee, Y. M., and Goodson, H. V. (2004) Molecular evolution of the histone deacetylase family: Functional implications of phylogenetic analysis. *Journal of Molecular Biology* **338**, 17-31
145. De Ruijter, A. J. M., Van Gennip, A. H., Caron, H. N., Kemp, S., and Van Kuilenburg, A. B. P. (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochemical Journal* **370**, 737-749
146. Haigis, M. C., and Guarente, L. P. (2006) Mammalian sirtuins - emerging roles in physiology, aging, and calorie restriction. *Genes & Development* **20**, 2913-2921
147. Brachmann, C. B., Sherman, J. M., Devine, S. E., Cameron, E. E., Pillus, L., and Boeke, J. D. (1995) THE SIR2 GENE FAMILY, CONSERVED FROM BACTERIA TO HUMANS, FUNCTIONS IN SILENCING, CELL-CYCLE PROGRESSION, AND CHROMOSOME STABILITY. *Genes & Development* **9**, 2888-2902
148. Morris, B. J. (2013) Seven sirtuins for seven deadly diseases of aging. *Free Radical Biology and Medicine* **56**, 133-171
149. Landry, J., Slama, J. T., and Sternglanz, R. (2000) Role of NAD(+) in the deacetylase activity of the SIR2-like proteins. *Biochemical and Biophysical Research Communications* **278**, 685-690
150. Tanner, K. G., Landry, J., Sternglanz, R., and Denu, J. M. (2000) Silent information regulator 2 family of NAD-dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 14178-14182
151. Tong, L., and Denu, J. M. (2010) Function and metabolism of sirtuin metabolite O-acetyl-ADP-ribose. *Biochimica Et Biophysica Acta-Proteins and Proteomics* **1804**, 1617-1625
152. Ahuja, N., Schwer, B., Carobbio, S., Waltregny, D., North, B. J., Castronovo, V., Maechler, P., and Verdin, E. (2007) Regulation of insulin secretion by SIRT4, a mitochondrial ADP-ribosyltransferase. *Journal of Biological Chemistry* **282**
153. Du, J. T., Zhou, Y. Y., Su, X. Y., Yu, J. J., Khan, S., Jiang, H., Kim, J., Woo, J., Kim, J. H., Choi, B. H., He, B., Chen, W., Zhang, S., Cerione, R. A., Auwerx, J., Hao,

Q., and Lin, H. N. (2011) Sirt5 Is a NAD-Dependent Protein Lysine Demalonylase and Desuccinylase. *Science* **334**, 806-809
154. Peng, C., Lu, Z. K., Xie, Z. Y., Cheng, Z. Y., Chen, Y., Tan, M. J., Luo, H., Zhang, Y., He, W., Yang, K., Zwaans, B. M. M., Tishkoff, D., Ho, L., Lombard, D., He, T. C., Dai, J. B., Verdin, E., Ye, Y., and Zhao, Y. M. (2011) The First Identification of Lysine Malonylation Substrates and Its Regulatory Enzyme. *Molecular & Cellular Proteomics* **10**
155. Liszt, G., Ford, E., Kurtev, M., and Guarente, L. (2005) Mouse Sir2 homolog SIRT6 is a nuclear ADP-ribosyltransferase. *Journal of Biological Chemistry* **280**, 21313-21320
156. Guedes-Dias, P., and Oliveira, J. M. A. (2013) Lysine deacetylases and mitochondrial dynamics in neurodegeneration. *Biochimica Et Biophysica Acta-Molecular Basis of Disease* **1832**, 1345-1359
157. Vaquero, A., Scher, M., Lee, D. H., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2004) Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin. *Molecular Cell* **16**, 93-105
158. Tanno, M., Sakamoto, J., Miura, T., Shimamoto, K., and Horio, Y. (2007) Nucleocytoplasmic shuttling of the NAD(+)-dependent histone deacrtylase SIRT1. *Journal of Biological Chemistry* **282**, 6823-6832
159. Hisahara, S., Chiba, S., Matsumoto, H., Tanno, M., Yagi, H., Shimohama, S., Sato, M., and Horio, Y. (2008) Histone deacetylase SIRT1 modulates neuronal differentiation by its nuclear translocation. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 15599-15604
160. Nemoto, S., Fergusson, M. M., and Finkel, T. (2005) SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1 alpha. *Journal of Biological Chemistry* **280**, 16456-16460
161. Fulco, M., and Sartorelli, V. (2008) Comparing and contrasting the roles of AMPK and SIRT1 in metabolic tissues. *Cell Cycle* **7**, 3669-3679
162. Cheng, H. L., Mostoslavsky, R., Saito, S., Manis, J. P., Gu, Y. S., Patel, P., Bronson, R., Appella, E., Alt, F. W., and Chua, K. F. (2003) Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 10794-10799
163. McBurney, M. W., Yang, X. F., Jardine, K., Hixon, M., Boekelheide, K., Webb, J. R., Lansdorp, P. M., and Lemieux, M. (2003) The mammalian SIR2 alpha protein has a role in embryogenesis and gametogenesis. *Molecular and Cellular Biology* **23**, 38-54
164. Rajendran, R., Garva, R., Krstic-Demonacos, M., and Demonacos, C. (2011) Sirtuins: Molecular Traffic Lights in the Crossroad of Oxidative Stress, Chromatin Remodeling, and Transcription. *Journal of Biomedicine and Biotechnology*
165. Vaquero, A., Scher, M. B., Lee, D. H., Sutton, A., Cheng, H. L., Alt, F. W., Serrano, L., Sternglanz, R., and Reinberg, D. (2006) SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis. *Genes & Development* **20**, 1256-1261
166. Voelter-Mahlknecht, S., Ho, A. D., and Mahlknecht, U. (2005) FISH-mapping and genomic organization of the NAD-dependent histone deacetylase gene, Sirtuin 2 (Sirt2). *International Journal of Oncology* **27**, 1187-1196
167. Maxwell, M. M., Tomkinson, E. M., Nobles, J., Wizeman, J. W., Amore, A. M., Quinti, L., Chopra, V., Hersch, S. M., and Kazantsev, A. G. (2011) The Sirtuin 2

microtubule deacetylase is an abundant neuronal protein that accumulates in the aging CNS. *Human Molecular Genetics* **20**, 3986-3996
168. Jin, Y. H., Kim, Y. J., Kim, D. W., Baek, K. H., Kang, B. Y., Yeo, C. Y., and Lee, K. Y. (2008) Sirt2 interacts with 14-3-3 beta/gamma and down-regulates the activity of p53. *Biochemical and Biophysical Research Communications* **368**, 690-695
169. Fernandez-Marcos, P. J., Jeninga, E. H., Canto, C., Harach, T., de Boer, V. C. J., Andreux, P., Moullan, N., Pirinen, E., Yamamoto, H., Houten, S. M., Schoonjans, K., and Auwerx, J. (2012) Muscle or liver-specific Sirt3 deficiency induces hyperacetylation of mitochondrial proteins without affecting global metabolic homeostasis. *Scientific Reports* **2**
170. Haigis, M. C., Mostoslavsky, R., Haigis, K. M., Fahie, K., Christodoulou, D. C., Murphy, A. J., Valenzuela, D. M., Yancopoulos, G. D., Karow, M., Blander, G., Wolberger, C., Prolla, T. A., Weindruch, R., Alt, F. W., and Guarente, L. (2006) SIRT4 inhibits glutamate dehydrogehase and opposes the effects of calorie restriction in pancreatic beta cells. *Cell* **126**, 941-954
171. He, W. J., Newman, J. C., Wang, M. Z., Ho, L., and Verdin, E. (2012) Mitochondrial sirtuins: regulators of protein acylation and metabolism. *Trends in Endocrinology and Metabolism* **23**, 467-476
172. Nakagawa, T., Lomb, D. J., Haigis, M. C., and Guarente, L. (2009) SIRT5 Deacetylates Carbamoyl Phosphate Synthetase 1 and Regulates the Urea Cycle. *Cell* **137**, 560-570
173. Jia, G. X., Su, L., Singhal, S., and Liu, X. G. (2012) Emerging roles of SIRT6 on telomere maintenance, DNA repair, metabolism and mammalian aging. *Molecular and Cellular Biochemistry* **364**, 345-350
174. Mostoslavsky, R., Chua, K. F., Lombard, D. B., Pang, W. W., Fischer, M. R., Gellon, L., Liu, P. F., Mostoslavsky, G., Franco, S., Murphy, M. M., Mills, K. D., Patel, P., Hsu, J. T., Hong, A. L., Ford, E., Cheng, H. L., Kennedy, C., Nunez, N., Bronson, R., Frendewey, D., Auerbach, W., Valenzuela, D., Karow, M., Hottiger, M. O., Hursting, S., Barrett, J. C., Guarente, L., Mulligan, R., Demple, B., Yancopoulos, G. D., and Alt, F. W. (2006) Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. *Cell* **124**, 315-329
175. Tennen, R. I., and Chua, K. F. (2011) Chromatin regulation and genome maintenance by mammalian SIRT6. *Trends in Biochemical Sciences* **36**, 39-46
176. Michishita, E., Park, J. Y., Burneskis, J. M., Barrett, J. C., and Horikawa, I. (2005) Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Molecular Biology of the Cell* **16**, 4623-4635
177. Ford, E., Voit, R., Liszt, G., Magin, C., GrumMt, I., and Guarente, L. (2006) Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription. *Genes & Development* **20**, 1075-1080
178. Barber, M. F., Michishita-Kioi, E., Xi, Y. X., Tasselli, L., Kioi, M., Moqtaderi, Z., Tennen, R. I., Paredes, S., Young, N. L., Chen, K. F., Struhl, K., Garcia, B. A., Gozani, O., Li, W., and Chua, K. F. (2012) SIRT7 links H3K18 deacetylation to maintenance of oncogenic transformation. *Nature* **487**, 114-+
179. Grob, A., Roussel, P., Wright, J. E., McStay, B., Hernandez-Verdun, D., and Sirri, V. (2009) Involvement of SIRT7 in resumption of rDNA transcription at the exit from mitosis. *Journal of Cell Science* **122**, 489-498
180. Vakhrusheva, O., Smolka, C., Gajawada, P., Kostin, S., Boettger, T., Kubin, T., Braun, T., and Bober, E. (2008) Sirt7 increases stress resistance of

cardiomyocytes and prevents apoptosis and inflammatory cardiomyopathy in mice. *Circulation Research* **102**, 703-710
181. Shvarts, A., Steegenga, W. T., Riteco, N., vanLaar, T., Dekker, P., Bazuine, M., vanHam, R. C. A., vanOordt, W. V., Hateboer, G., vanderEb, A. J., and Jochemsen, A. G. (1996) MDMX: A novel p53-binding protein with some functional properties of MDM2. *Embo Journal* **15**, 5349-5357
182. Jackson, M. W., and Berberich, S. J. (2000) MdmX protects p53 from Mdm2-mediated degradation. *Molecular and Cellular Biology* **20**, 1001-1007
183. Tanimura, S., Ohtsuka, S., Mitsui, K., Shirouzu, K., Yoshimura, A., and Ohtsubo, M. (1999) MDM2 interacts with MDMX through their RING finger domains. *Febs Letters* **447**, 5-9
184. Sharp, D. A., Kratowicz, S. A., Sank, M. J., and George, D. L. (1999) Stabilization of the MDM2 oncoprotein by interaction with the structurally related MDMX protein. *Journal of Biological Chemistry* **274**, 38189-38196
185. Gu, J. J., Kawai, H., Nie, L. G., Kitao, H., Wiederschain, D., Jochemsen, A. G., Parant, J., Lozano, G., and Yuan, Z. M. (2002) Mutual dependence of MDM2 and MDMX in their functional inactivation of p53. *Journal of Biological Chemistry* **277**, 19251-19254
186. Linke, K., Mace, P. D., Smith, C. A., Vaux, D. L., Silke, J., and Day, C. L. (2008) Structure of the MDM2/MDMX RING domain heterodimer reveals dimerization is required for their ubiquitylation in trans. *Cell Death and Differentiation* **15**, 841-848
187. Deshaies, R. J., and Joazeiro, C. A. P. (2009) RING Domain E3 Ubiquitin Ligases. *Annual Review of Biochemistry* **78**, 399-434
188. Schuller, M., Jenne, D., and Voltz, R. (2005) The human PNMA family: Novel neuronal proteins implicated in paraneoplastic neurological disease. *Journal of Neuroimmunology* **169**, 172-176
189. Fang, S. Y., Jensen, J. P., Ludwig, R. L., Vousden, K. H., and Weissman, A. M. (2000) Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *Journal of Biological Chemistry* **275**, 8945-8951
190. Diaz-Griffero, F., Li, X., Javanbakht, H., Song, B., Welikala, S., Stremlau, M., and Sodroski, J. (2006) Rapid turnover and polyubiquitylation of the retroviral restriction factor TRIM5. *Virology* **349**, 300-315
191. Kaiser, P., Seufert, W., Hofferer, L., Kofler, B., Sachsenmaier, C., Herzog, H., Jentsch, S., Schweiger, M., and Schneider, R. (1994) A HUMAN UBIQUITIN-CONJUGATING ENZYME HOMOLOGOUS TO YEAST UBC8. *Journal of Biological Chemistry* **269**, 8797-8802
192. Bothos, J., Summers, M. K., Venere, M., Scolnick, D. M., and Halazonetis, T. D. (2003) The Chfr mitotic checkpoint protein functions with Ubc13-Mms2 to form Lys63-linked polyubiquitin chains. *Oncogene* **22**, 7101-7107
193. Plans, V., Scheper, J., Soler, M., Loukili, N., Okano, Y., and Thomson, T. M. (2006) The RING finger protein RNF8 recruits UBC13 for lysine 63-based self polyubiquitylation. *Journal of Cellular Biochemistry* **97**, 572-582
194. Saville, M. K., Sparks, A., Xirodimas, D. P., Wardrop, J., Stevenson, L. F., Bourdon, J. C., Woods, Y. L., and Lane, D. P. (2004) Regulation of p53 by the ubiquitin-conjugating enzymes UbcH5B/C in vivo. *Journal of Biological Chemistry* **279**, 42169-42181
195. David, Y., Ziv, T., Admon, A., and Navon, A. (2010) The E2 Ubiquitin-conjugating Enzymes Direct Polyubiquitination to Preferred Lysines. *Journal of Biological Chemistry* **285**, 8595-8604

196. Semizarov, D., Frost, L., Sarthy, A., Kroeger, P., Halbert, D. N., and Fesik, S. W. (2003) Specificity of short interfering RNA determined through gene expression signatures. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 6347-6352
197. Persengiev, S. P., Zhu, X. C., and Green, M. R. (2004) Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). *Rna-a Publication of the Rna Society* **10**, 12-18
198. Kawai, T., and Akira, S. (2011) Regulation of innate immune signalling pathways by the tripartite motif (TRIM) family proteins. *Embo Molecular Medicine* **3**, 513-527
199. McNab, F. W., Rajsbaum, R., Stoye, J. P., and O'Garra, A. (2011) Tripartite-motif proteins and innate immune regulation. *Current Opinion in Immunology* **23**, 46-56
200. Lee, Y., Song, B., Park, C., and Kwon, K. S. (2013) TRIM11 Negatively Regulates IFN beta Production and Antiviral Activity by Targeting TBK1. *Plos One* **8**
201. Uchil, P. D., Quinlan, B. D., Chan, W. T., Luna, J. M., and Mothes, W. (2008) TRIM E3 ligases interfere with early and late stages of the retroviral life cycle. *Plos Pathogens* **4**
202. Niikura, T., Hashimoto, Y., Tajima, H., Ishizaka, M., Yamagishi, Y., Kawasumi, M., Nawa, M., Terashita, K., Aiso, S., and Nishimoto, I. (2003) A tripartite motif protein TRIM11 binds and destabilizes Humanin, a neuroprotective peptide against Alzheimer's disease-relevant insults. *European Journal of Neuroscience* **17**, 1150-1158
203. Ishikawa, H., Tachikawa, H., Miura, Y., and Takahashi, N. (2006) TRIM11 binds to and destabilizes a key component of the activator-mediated cofactor complex (ARC105) through the ubiquitin-proteasome system. *Febs Letters* **580**, 4784-4792
204. Tuoc, T. C., and Stoykova, A. (2008) Trim11 modulates the function of neurogenic transcription factor Pax6 through ubiquitin-proteosome system. *Genes & Development* **22**, 1972-1986
205. Parodi, S., Di Zanni, E., Di Lascio, S., Bocca, P., Prigione, I., Fornasari, D., Pennuto, M., Bachetti, T., and Ceccherini, I. (2012) The E3 ubiquitin ligase TRIM11 mediates the degradation of congenital central hypoventilation syndrome-associated polyalanine-expanded PHOX2B. *Journal of Molecular Medicine-Jmm* **90**, 1025-1035
206. Sasaki, T., Maier, B., Koclega, K. D., Chruszcz, M., Gluba, W., Stukenberg, P. T., Minor, W., and Scrable, H. (2008) Phosphorylation Regulates SIRT1 Function. *Plos One* **3**
207. North, B. J., and Verdin, E. (2007) Mitotic regulation of SIRT2 by cyclin-dependent kinase 1-dependent phosphorylation. *Journal of Biological Chemistry* **282**, 19546-19555
208. Fantz, D. A., Jacobs, D., Glossip, D., and Kornfeld, K. (2001) Docking sites on substrate proteins direct extracellular signal-regulated kinase to phosphorylate specific residues. *Journal of Biological Chemistry* **276**, 27256-27265
209. Okazaki, T., Kato, Y., Mochizuki, T., Tashima, M., Sawada, H., and Uchino, H. (1988) STAUROSPORINE, A NOVEL PROTEIN-KINASE INHIBITOR, ENHANCES HL-60-CELL DIFFERENTIATION INDUCED BY VARIOUS COMPOUNDS. *Experimental Hematology* **16**, 42-48

210. Lawrie, A. M., Noble, M. E. M., Tunnah, P., Brown, N. R., Johnson, L. N., and Endicott, J. A. (1997) Protein kinase inhibition by staurosporine revealed in details of the molecular interaction with CDK2. *Nature Structural Biology* **4**, 796-801
211. Cagnol, S., and Chambard, J. C. (2010) ERK and cell death: Mechanisms of ERK-induced cell death - apoptosis, autophagy and senescence. *Febs Journal* **277**, 2-21
212. Tsuruta, F., Masuyama, N., and Gotoh, Y. (2002) The phosphatidylinositol 3-kinase (PI3K)-Akt pathway suppresses Bax translocation to mitochondria. *Journal of Biological Chemistry* **277**, 14040-14047
213. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Cellular survival: a play in three Akts. *Genes & Development* **13**, 2905-2927
214. Tibbles, L. A., and Woodgett, J. R. (1999) The stress-activated protein kinase pathways. *Cellular and Molecular Life Sciences* **55**, 1230-1254
215. Young, P. R., McLaughlin, M. M., Kumar, S., Kassis, S., Doyle, M. L., McNulty, D., Gallagher, T. F., Fisher, S., McDonnell, P. C., Carr, S. A., Huddleston, M. J., Seibel, G., Porter, T. G., Livi, G. P., Adams, J. L., and Lee, J. C. (1997) Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. *Journal of Biological Chemistry* **272**, 12116-12121
216. Hirt, L., Badaut, J., Thevenet, J., Granziera, C., Regli, L., Maurer, F., Bonny, C., and Bogousslavsky, J. (2004) D-JNKI1,a cell-penetrating c-Jun-N-terminal kinase inhibitor, protects against cell death in severe cerebral ischemia. *Stroke* **35**, 1738-1743
217. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) PD-098059 IS A SPECIFIC INHIBITOR OF THE ACTIVATION OF MITOGEN-ACTIVATED PROTEIN-KINASE KINASE IN-VITRO AND IN-VIVO. *Journal of Biological Chemistry* **270**, 27489-27494
218. Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F., Copeland, R. A., Magolda, R. L., Scherle, P. A., and Trzaskos, J. M. (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *Journal of Biological Chemistry* **273**, 18623-18632
219. Wymann, M. P., BulgarelliLeva, G., Zvelebil, M. J., Pirola, L., Vanhaesebroeck, B., Waterfield, M. D., and Panayotou, G. (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Molecular and Cellular Biology* **16**, 1722-1733
220. Yuan, T. L., and Cantley, L. C. (2008) PI3K pathway alterations in cancer: variations on a theme. *Oncogene* **27**, 5497-5510
221. Stein, R. C., and Waterfield, M. D. (2000) PI3-kinase inhibition: a target for drug development? *Molecular Medicine Today* **6**, 347-357
222. Walker, E. H., Pacold, M. E., Perisic, O., Stephens, L., Hawkins, P. T., Wymann, M. P., and Williams, R. L. (2000) Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Molecular Cell* **6**, 909-919
223. Oshiro, N., Yoshino, K., Hidayat, S., Tokunaga, C., Hara, K., Eguchi, S., Avruch, J., and Yonezawa, K. (2004) Dissociation of raptor from mTOR is a mechanism of rapamycin-induced inhibition of mTOR function. *Genes to Cells* **9**, 359-366
224. Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003) The specificities of protein kinase inhibitors: an update. *Biochemical Journal* **371**, 199-204

225. Vogt, P. K., Gymnopoulos, M., and Hart, J. R. (2009) PI 3-kinase and cancer: changing accents. *Current Opinion in Genetics & Development* **19**, 12-17
226. Collins, I., and Workman, P. (2006) New approaches to molecular cancer therapeutics. *Nature Chemical Biology* **2**, 689-700
227. Workman, P. (2007) Drugging the cancer genome: New challenges of infrequent and combinatorial targets. *Current Opinion in Investigational Drugs* **8**, 445-446
228. Workman, P., Clarke, P. A., Raynaud, F. I., and van Montfort, R. L. M. (2010) Drugging the PI3 Kinome: From Chemical Tools to Drugs in the Clinic. *Cancer Research* **70**, 2146-2157
229. Knight, Z. A., and Shokat, K. M. (2007) Chemically targeting the PI3K family. *Biochemical Society Transactions* **35**, 245-249
230. Knight, Z. A., Chiang, G. G., Alaimo, P. J., Kenski, D. M., Ho, C. B., Coan, K., Abraham, R. T., and Shokat, K. M. (2004) Isoform-specific phosphoinositide 3-kinase inhibitors from an arylmorpholine scaffold. *Bioorganic & Medicinal Chemistry* **12**, 4749-4759
231. Knight, Z. A., Gonzalez, B., Feldman, M. E., Zunder, E. R., Goldenberg, D. D., Williams, O., Loewith, R., Stokoe, D., Balla, A., Toth, B., Balla, T., Weiss, W. A., Williams, R. L., and Shokat, K. M. (2006) A pharmacological map of the PI3-K family defines a role for p110 alpha in insulin signaling. *Cell* **125**, 733-747
232. Park, S., Chapuis, N., Bardet, V., Tamburini, J., Gallay, N., Willems, L., Knight, Z. A., Shokat, K. M., Azar, N., Viguie, F., Ifrah, N., Dreyfus, F., Mayeux, P., Lacombe, C., and Bouscary, D. (2008) PI-103, a dual inhibitor of Class IA phosphatidylinositide 3-kinase and mTOR, has antileukemic activity in AML. *Leukemia* **22**, 1698-1706
233. Fan, Q. W., Knight, Z. A., Goldenberg, D. D., Yu, W., Mostov, K. E., Stokoe, D., Shokat, K. M., and Weiss, W. A. (2006) A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. *Cancer Cell* **9**, 341-349
234. Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C. J., McLauchlan, H., Klevernic, I., Arthur, J. S. C., Alessi, D. R., and Cohen, P. (2007) The selectivity of protein kinase inhibitors: a further update. *Biochemical Journal* **408**, 297-315
235. Downey, G. P., Butler, J. R., Brumell, J., Borregaard, N., Kjeldsen, L., SueAquan, A. K., and Grinstein, S. (1996) Chemotactic peptide-induced activation of MEK-2, the predominant isoform in human neutrophils - Inhibition by wortmannin. *Journal of Biological Chemistry* **271**, 21005-21011
236. Odwyer, P. J., Leylandjones, B., Alonso, M. T., Marsoni, S., and Wittes, R. E. (1985) ETOPOSIDE (VP-16-213) - CURRENT STATUS OF AN ACTIVE ANTICANCER DRUG. *New England Journal of Medicine* **312**, 692-700
237. Tang, D. M., Wu, D. C., Hirao, A., Lahti, J. M., Liu, L. Q., Mazza, B., Kidd, V. J., Mak, T. W., and Ingram, A. J. (2002) ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. (vol 277, pg 12710, 2002). *Journal of Biological Chemistry* **277**, 21110-21110
238. Huang, W. D., and Erikson, R. L. (1994) CONSTITUTIVE ACTIVATION OF MEK1 BY MUTATION OF SERINE PHOSPHORYLATION SITES. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 8960-8963
239. Pan, Y., and Chen, J. D. (2003) MDM2 promotes ubiquitination and degradation of MDMX. *Molecular and Cellular Biology* **23**, 5113-5121
240. Goping, I. S., Gross, A., Lavoie, J. N., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S. J., and Shore, G. C. (1998) Regulated targeting of BAX to mitochondria. *Journal of Cell Biology* **143**, 207-215

241. Lin, H. K., Wang, L., Hu, Y. C., Altuwaijri, S., and Chang, C. S. (2002) Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. *Embo Journal* **21**, 4037-4048
242. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) A SYNTHETIC INHIBITOR OF THE MITOGEN-ACTIVATED PROTEIN-KINASE CASCADE. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 7686-7689
243. Tentler, J. J., Nallapareddy, S., Tan, A. C., Spreafico, A., Pitts, T. M., Morelli, M. P., Selby, H. M., Kachaeva, M. I., Flanigan, S. A., Kulikowski, G. N., Leong, S., Arcaroli, J. J., Messersmith, W. A., and Eckhardt, S. G. (2010) Identification of Predictive Markers of Response to the MEK1/2 Inhibitor Selumetinib (AZD6244) in K-ras-Mutated Colorectal Cancer. *Molecular Cancer Therapeutics* **9**, 3351-3362
244. Tamura, Y., Simizu, S., and Osada, H. (2004) The phosphorylation status and anti-apoptotic activity of Bcl-2 are regulated by ERK and protein phosphatase 2A on the mitochondria. *Febs Letters* **569**, 249-255
245. Bonni, A., Brunet, A., West, A. E., Datta, S. R., Takasu, M. A., and Greenberg, M. E. (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* **286**, 1358-1362
246. Imai, S., Armstrong, C. M., Kaeberlein, M., and Guarente, L. (2000) Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* **403**, 795-800
247. Houtkooper, R. H., Canto, C., Wanders, R. J., and Auwerx, J. (2010) The Secret Life of NAD(+): An Old Metabolite Controlling New Metabolic Signaling Pathways. *Endocrine Reviews* **31**, 194-223
248. Houtkooper, R. H., and Auwerx, J. (2012) Exploring the therapeutic space around NAD(+). *Journal of Cell Biology* **199**, 205-209
249. (!!! INVALID CITATION !!!).
250. Byles, V., Chmilewski, L. K., Wang, J., Zhu, L. J., Forman, L. W., Faller, D. V., and Dai, Y. (2010) Aberrant Cytoplasm Localization and Protein Stability of SIRT1 is Regulated by PI3K/IGF-1R Signaling in Human Cancer Cells. *International Journal of Biological Sciences* **6**, 599-612
251. Pfister, J. A., Ma, C., Morrison, B. E., and D'Mello, S. R. (2008) Opposing Effects of Sirtuins on Neuronal Survival: SIRT1-Mediated Neuroprotection Is Independent of Its Deacetylase Activity. *Plos One* **3**
252. Shin, J. Y., Zhang, D., and Chen, D. (2011) Reversible Acetylation of Metabolic Enzymes Celebration: SIRT2 and p300 Join the Party. *Molecular Cell* **43**, 3-5
253. Liu, Y., Dentin, R., Chen, D., Hedrick, S., Ravnskjaer, K., Schenk, S., Milne, J., Meyers, D. J., Cole, P., Yates, J., Olefsky, J., Guarente, L., and Montminy, M. (2008) A fasting inducible switch modulates gluconeogenesis via activator/coactivator exchange. *Nature* **456**, 269-U274
254. Haigis, M. C., and Sinclair, D. A. (2010) Mammalian Sirtuins: Biological Insights and Disease Relevance. *Annual Review of Pathology-Mechanisms of Disease* **5**, 253-295
255. Phizicky, E. M., and Fields, S. (1995) PROTEIN-PROTEIN INTERACTIONS - METHODS FOR DETECTION AND ANALYSIS. *Microbiological Reviews* **59**, 94-123
256. Shimazu, T., Hirschey, M. D., Hua, L., Dittenhafer-Reed, K. E., Schwer, B., Lombard, D. B., Li, Y., Bunkenborg, J., Alt, F. W., Denu, J. M., Jacobson, M. P.,

and Verdin, E. (2010) SIRT3 Deacetylates Mitochondrial 3-Hydroxy-3-Methylglutaryl CoA Synthase 2 and Regulates Ketone Body Production. *Cell Metabolism* **12**, 654-661
257. Liu, B., Che, W. L., Zheng, C. Z., Liu, W. J., Wen, J., Fu, H. T., Tang, K., Zhang, J. Y., and Xu, Y. W. (2013) SIRTS: A Safeguard Against Oxidative Stress-Induced Apoptosis in Cardiomyocytes. *Cellular Physiology and Biochemistry* **32**, 1050-1059
258. Roskoski, R. (2012) MEK1/2 dual-specificity protein kinases: Structure and regulation. *Biochemical and Biophysical Research Communications* **417**, 5-10
259. Letai, A. (2006) Growth factor withdrawal and apoptosis: The middle game. *Molecular Cell* **21**, 728-730
260. Hong, S. J., Chae, H., Lardaro, T., Hong, S., and Kim, K. S. (2008) Trim11 increases expression of dopamine beta-hydroxylase gene by interacting with Phox2b. *Biochemical and Biophysical Research Communications* **368**, 650-655
261. Napolitano, L. M., and Meroni, G. (2012) TRIM family: Pleiotropy and diversification through homomultimer and heteromultimer formation. *Iubmb Life* **64**, 64-71
262. Yamauchi, K., Wada, K., Tanji, K., Tanaka, M., and Kamitani, T. (2008) Ubiquitination of E3 ubiquitin ligase TRIM5 alpha and its potential role. *Febs Journal* **275**, 1540-1555
263. McDowell, G. S., and Philpott, A. (2013) Non-canonical ubiquitylation: Mechanisms and consequences. *International Journal of Biochemistry & Cell Biology* **45**, 1833-1842
264. Jing, E. X., Gesta, S., and Kahn, C. R. (2007) SIRT2 regulates adipocyte differentiation through FoxO1 acetylation/deacetylation. *Cell Metabolism* **6**, 105-114
265. Haussinger, D. (1990) NITROGEN-METABOLISM IN LIVER - STRUCTURAL AND FUNCTIONAL-ORGANIZATION AND PHYSIOLOGICAL RELEVANCE. *Biochemical Journal* **267**, 281-290
266. Meijer, A. J., Lamers, W. H., and Chamuleau, R. (1990) NITROGEN-METABOLISM AND ORNITHINE CYCLE FUNCTION. *Physiological Reviews* **70**, 701-748